Leukotriene E4 receptors in bronchial asthma

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Awarding institution:
King's College London

Leukotriene E_4 receptors in bronchial asthma

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Submitted for the degree of

Doctor of Philosophy

at King's College London

MRC & Asthma UK Centre in Allergic Mechanisms of Asthma

Division of Asthma, Allergy and Lung Biology

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2014

Abstract

Cysteinyl leukotriene (LTC_4, LTD_4 and LTE_4) are proinflammatory lipid mediators involved in the pathogenesis of asthma. LTE_4 the most stable of the cysteinyl leukotrienes elicits bronchoconstriction, and unlike LTC_4 and LTD_4 it enhances bronchial hyperresponsiveness. LTE_4 can also mediate other features of asthma, such as eosinophilia, increased mucus secretion and vascular permeability. None of these LTE_4 activities can be explained by current understanding of classical cysteinyl leukotriene receptors, $CysLT_1$ and $CysLT_2$, suggesting the presence of a novel receptor that preferentially responds to LTE_4.

LTE_4 mediated pulmonary inflammation in a murine model was shown to remain intact in the absence of $CysLT_1$ and $CysLT_2$, and was abrogated by the knockout of the purinergic receptor, $P2Y_{12}$. This study aimed to elucidate whether LTE_4 directly signalled through the $P2Y_{12}$ receptor. Models of human $CysLT_1$, $CysLT_2$ and $P2Y_{12}$ overexpressed in HEK293T, CHO cells and human platelets were used to characterise responsiveness to cysteinyl leukotrienes. LTE_4 failed to induce any specific signalling responses in cells expressing $P2Y_{12}$ or in human platelets, showing that LTE_4 does not directly signal through $P2Y_{12}$.

Previous reports have shown that LTE_4 is a potent agonist in human mast cells and so the aim was to identify the receptor responsible for LTE_4-induced responses using two human mast cell lines, LAD2 and LUVA. When intracellular signalling and gene expression were compared in response to stimulation, LUVA cells responded to LTC_4 and LTD_4 but lacked the potent responses to LTE_4 observed in LAD2 cells. Gene screening by microarrays was used to identify 4 target GPCRs which were cloned and their responsiveness to cysteinyl leukotrienes analysed in HEK293T overexpression model. $CysLT_1$ was identified as the only GPCR responding to cysteinyl leukotrienes and differently expressed in LAD2 cells. Stable knockdowns were created and analysed to reveal $CysLT_1$ was responsible for the augmented responses to LTE_4 observed in LAD2 cells.

Acknowledgments

First and foremost, I would like to thank my PhD supervisor Dr Grzegorz Woszczek for giving me the opportunity to do research within his lab. He has kept me motivated throughout these 4 years with his support, guidance and immense knowledge, which taken together have made him a great mentor. I am particular grateful for his patience, especially with taking on a student who had no prior biological lab experience. I must have been a bit tedious at times but I thank him for sticking with me.

I want to thank my secondary supervisors Professor Christopher Corrigan and Dr James Pease for their continued support of my research project and especially for their advice while I was writing up. I would also like to thank Dr Elisabeth Fürst for everything that she has taught me within the lab. Her support and guidance has been invaluable.

To my department, AARS, my time at KCL has been so rewarding not only because I was doing something that I enjoyed but because of the people I was able to work with. I would specifically like to thank Dave Richards. He has helped me more times than I can remember and my day-to-day lab work would not have run as smoothly as it did if it wasn't for him. Thanks to Emma Chambers (the FACS-master and former desk buddy), Tom Pagdin, Sarah Dimeloe and Celine Wu for their support and friendship. For the Friday evening pub trips, festivals and amazing holidays together. To my office neighbours, Batika Rana and Winston Lu, I cannot think of two better people to have shared an office with. Thanks to Winston for organising our work badminton sessions (badAARS), for the random evening philosophical quandaries and for eating all my food! Thanks to Batika, for being a true friend, for being so supportive and fun; from the odd hot chocolate on the 16th floor to all our cinema trips, random evenings of cribbage and port, and the slightly stressful (but well recovered) jaunt to Italy. I would also like to thank the people I have shared a lab with, Charles Mackay, David Wright, Rebekah Sherburn and Yasin Shaifta for making such a great work environment. I am particularly grateful to Charlie for keeping me sane within the last year of labs and for all the random chats (which I apologise for).

I would also like to specifically thank two friends who were not part of AARS. To Naomi Peirson and William Holland. I am very glad to have met Na at university because I not only gained one amazing friend, but also another in the form of her brother Will. I thank them for their support and honest advice during my entire Phd.

Finally, I would like to dedicate this thesis to my family; to my parents, Mat, Laura, Harry, Nan and Taran. They have unconditionally supported me throughout my life and I am very grateful to them. I love them dearly and know that without them I would not be where I am today. Thank you.

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Abbreviations

2APB	2-aminoethoxydiphenyl borate
2-MeS-ADP	2-methylthio-adenosine-5'-diphosphate
3'UTR	3' untranslated region
5-LO	5-lipoxygenase
ACD	citrate-dextrose solution
ADP	adenosine-5'-diphosphate
AERD	aspirin-exacerbated respiratory disease
Akt	protein kinase B
ANOVA	analysis of variance
AP-2	adapter protein 2
ASM	airway smooth muscle
ATP	adenosine triphosphate
ATP	adenosine-5'-triphosphate
BAL	bronchial alveolar lavage fluid
BCA	bicinchoninic acid assay
bp	base pair
cAMP	adenosine 3,5-monophosphate
cAMP-GEFI	cAMP-regulated guanine nucleotide exchange factors 1
cAMP-GEFII	cAMP-regulated guanine nucleotide exchange factors 2
CDS	coding region
CHO	Chinese hamster ovary cells
COOH	carboxyl-
COPD	chronic obstructive pulmonary disease
COX	cyclooxygenase

CPI-17	myosin phosphatase inhibitor
$cPLA_2$	cytosolic phospholipase A_2
CREB	cAMP responsive element binding protein
cRNA	complementary RNA
Ct	cycle threshold
C-terminus	carboxy-terminus
Ctl.	vehicle control
DAG	diacylglycerol
DCs	dendritic cells
DMEM	Dulbecco's modified Eagle's medium
DP	dipeptidase
EDTA	ethylenediaminetetraacetic acid
EET	epioxyeicosatrienoic acid
Egr-3	early growth response protein-3
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPACs	exchange proteins activated by cAMP
EtOH	ethanol
FLAP	5-lipoxgenase-activating protein
γ-GT	γ-glutamyl transpeptidase
GDP	guanosine diphosphate
GFP	green fluorescent protein
GMCSF	granulocyte-macrophage colony-stimulating factor
GPCR	G-protein coupled receptor
G-protein	guanine nucleotide-binding protein

GRKs	GPCR kinases
GSK3β	glycogen synthase kinase 3β
GTP	guanosine triphosphate
h-	human
HA	hemagglutinin
h-ADRβ_2	human β_2 adrenergic receptor
HAMI	HAMI 3379
HBS	HEPES buffered saline
HCl	hydrochloric acid
HEK293T	human embryonic kidney 293T
HETE	hydroxyeicosatetraenoic acid
HFL-1	human foetal lung fibroblasts
HL-60	myeloid leukaemia cell line
HRP	horseradish peroxidase
Hs	*Homo sapiens*
HUVEC	human umbilical vein endothelial cells
IBMX	3-isobutyl-1-methylxanthine
IFN-γ	interferon-γ
IGF-1	insulin growth factor-1
IP_3	inositol-1,4,5-triphosphate
IUPHAR	International Union of Pharmacology
JNKs	c-Jun N terminal kinases
K^+	potassium
KCl	potassium chloride
KD	knockdown

LTA_4	leukotriene A_4
LTA_4H	LTA_4 hydrolase
LTB_4	leukotriene B_4
LTC_4	leukotriene C_4
LTC_4S	LTC_4 synthase
LTD_4	leukotriene D_4
LTE_4	leukotriene E_4
LTRAs	cysteinyl leukotriene receptor antagonists
Mac-1	macrophage adhesion ligand-1
MAPK	mitogen-activated protein kinase
$MgCl_2$	magnesium chloride
MK	MK-571
Mm	*Mus musculus*
Mont	Montelukast
mRNA	messenger RNA
MRP1	multidrug resistance-associated proteins 1
MRP4	multidrug resistance-associated proteins 4
MS	multiple sclerosis
NaCl	sodium chloride
NCBI	National Centre for Biotechnology Information
NH_2	amino-
PAF	platelet-activating factor
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDZ-GEFI	PDZ-guanine nucleotide exchange factor 1

PEG	polyethylene glycol
PGE_1	prostaglandin E_1
PIP_2	phosphatidylinositol-4,5-biphosphate
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PRP	platelet-rich plasma
Ptx	pertussis toxin
qRT-PCR	quantitative real time polymerase chain reaction
RFU	relative fluorescence units
RI	relative Intensity
RLU	relative luminescence units
rRNA	ribosomal RNA
SCF	stem cell factor
SD	standard deviation
SEM	standard error of the mean
shRNA	small hairpin RNA
siRNA	small interfering RNA
SNPs	single nucleotide polymorphisms
SRS-A	slow-reacting substance of anaphylaxis
TAE	tris-acetate-EDTA
TBS	tris buffered saline
TMs	transmembrane domains
TTBS	tris buffered saline 0.05 % Tween

UDP	uridine-5’-diphosphate
UDP	uridine-5’-diphosphate
UV	ultraviolet
VCAM-1	vascular cell adhesion molecule-1

Chapter 1

Introduction

Asthma is a disorder that affects the airways of 5.4 million people in the UK, of which over a million are children. Once thought to be a disease of the airway smooth muscle, it is now defined as an inflammatory disease of the conducting airways, which gives rise to specific structural and functional changes. It is considered a heterogeneous, multi-factorial condition in which not one single gene or environmental factor can be held accountable. Instead it is potentially caused by the interplay of several of these factors.

In this introduction I will be focussing on the involvement of specific proinflammatory mediators, known as cysteinyl leukotrienes, which are thought to play critical roles in many of the key pathways of the pathogenesis of asthma. I will first introduce the discovery and synthesis of these mediators and intracellular signalling pathways that are mediated by one of the largest classes of receptors, known as G-protein coupled receptors (GPCRs). I will review in detail cysteinyl leukotrienes involvement in asthma with particular emphasis on leukotriene E_4 (LTE_4), the most stable of the cysteinyl leukotrienes and potentially the most important leukotriene in terms of pathogenesis of the disease. Finally, I will discuss the importance of LTE_4 and potential existence of a previously unidentified cysteinyl leukotriene receptor.

1.1.1 Lipid mediators

Lipids are essential molecules for life. They make up the majority of all cell membranes as phospholipids and are crucial energy sources. They can also act as signalling molecules (lipid mediators) which can directly play pivotal roles in inflammation, immunity and homeostasis. Lipid mediators can be distinguished from other signalling molecules such as neurotransmitters and hormones by their ability to be produced on demand in a calcium-dependent fashion from membrane phospholipids. Lipid mediators are also able to activate a specific group of receptors, known as GPCRs. Thus lipid mediator generation and signalling are highly regulated and so any dysfunction through synthesis or receptor can cause and potentiate disease states. This has led to a plethora of enzyme inhibitors and receptor antagonists being developed for use as therapeutic targets, especially in the field of allergic disorders (namely asthma).

Leukotrienes, prostanoids (prostaglandins and thromboxanes), hydroxyeicosatetraenoic acids, lipoxins and epoxyeicosatrienoic acid are collectively known as eicosanoids. They are a large and ever-growing family of lipid mediators that are structurally distinct from other lipids as they made up of fatty acid chains that are 20 carbons long (“eicosa” in Greek means 20), and their synthesis is the result of the enzymatic metabolism by phospholipase A_2 (PLA_2) of a precursor molecule known as arachidonic acid (Figure 1.1).

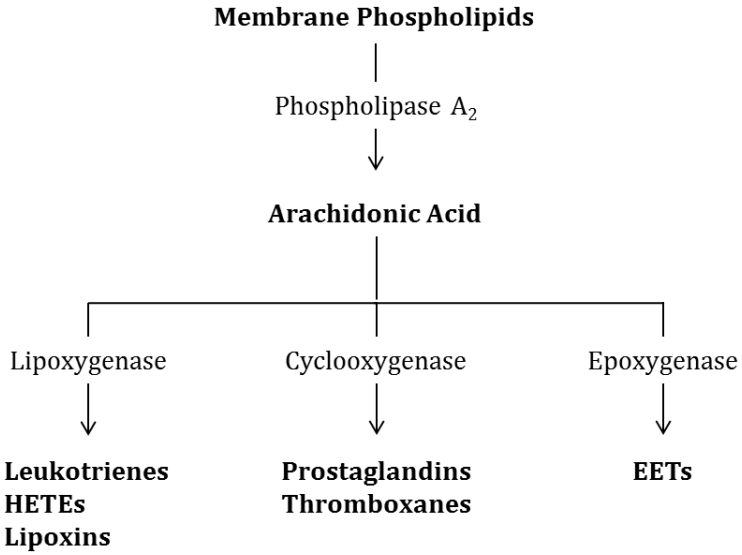


Figure 1.1 Overview of eicosanoid synthesis from precursor, arachidonic acid.

Phospholipase A_2 metabolises membrane phospholipids to arachidonic acid. Arachidonic acid can be further metabolised by lipoxygenase to produce leukotrienes, hydroxyeicosatetraenoic acids (HETEs) and lipoxins; by cyclooxygenase to produce prostaglandins and thromboxanes; by epoxygenase to produce epioxyeicosatrienoic acids (EETs).

PLA_2s are enzymes that prompt the de-esterification of phospholipids in order to yield lysophospholipids and arachidonic acid. They are abundantly expressed in humans and are utilised upon certain pathological and physiological stimuli. There are more than 20 different PLA_2s in mammals and these are divided into four groups. Secretory, calcium-independent, platelet-activating factor (PAF) acetylhydrolyases, and cytosolic. Cytosolic PLA_2 ($cPLA_2$) has been shown to be the most important for eicosanoid production (1). They localise in the cytoplasm and upon cell activation, translocate to the nuclear membrane in a calcium-dependent manner. Despite calcium being essential for $cPLA_2$ functionality it is not required for the catalytic activity, unlike secretory PLA_2s (2). At the nuclear membrane $cPLA_2$ hydrolyses phospholipids in the *sn*-2 position (second carbon) with a specific preference for yielding arachidonic acid (1). Free arachidonic acid can then react with various enzymes to yield the metabolites known as eicosanoids (shown in Figure 1.1). Lipoxygenases produce leukotrienes, hydroxyeicosatetraenoic acids (HETEs) and lipoxins. Cyclooxygenases produce prostanoids (prostaglandins and thromboxanes) and finally epoxygenases, more specifically cytochrome P-450, produces epioxyeicosatrienoic acids (EETs). All eicosanoids can activate GPCRs which in the case of

cysteinyl leukotrienes can lead to various biological responses that have been implicated in many diseases such as asthma, atherosclerosis and cancer.

1.1.2 G-protein coupled receptors and signal transduction

GPCRs are the largest and most versatile family of transmembrane proteins within humans. They can mediate extracellular signals via intracellular interactions with heterotrimeric guanine nucleotide-binding proteins (G-proteins) to induce changes within the cell. This affects enzymes, ion channels and regulation of gene expression. GPCR signalling is critical in many physiological processes. These include senses (taste, sight and smell), neurological, immunological, cardiovascular and reproductive functions and so it is not surprising that they make up nearly half of the molecular targets in the current market of therapeutic drugs (3). There have been several milestones in GPCR research, one of which being the sequencing of the human genome. This revealed that the GPCR family (or superfamily) contains more than 800 members, of which approximately 460 are predicted to be olfactory receptors. A large proportion of the GPCR superfamily currently have unknown functionality and are termed orphan receptors. Characterising these orphan receptors represents an interesting group of targets for pharmaceutical companies.

The GPCR superfamily has been divided into 5 families based on amino acid sequence (to avoid confusion with GPCRs of similar names GPCR families will be denoted in italics); *Rhodopsin* (~700 GPCRs), *Secretin* (15 GPCRs), *Glutamate* (15 GPCRs), *Adhesion* (24 GPCRs) and *Frizzled/Taste* family (24 GPCRs) (3). All five GPCR families share little amino acid sequence homology even in the *Rhodopsin* family, subfamilies α, β, γ and δ, share only ≥25 % sequence homology, with homologies being slightly higher within these subfamilies (≥30 %).

GPCR structure

X-ray crystallography has been a major development in GPCR research especially for structural determination. In 2000, bovine rhodopsin was the first GPCR to have its structure resolved (4). Up until 2007, GPCR structural research was focussed on computer modelling based on rhodopsin. Due to novel protein crystallisation approaches and wider access to x-ray crystallography, an almost exponential boom in resolved GPCR structures has been reported. Currently, there are just over 20 GPCRs with resolved structures, in which over two-thirds of them were published between 2012 and 2014 (Table 1.1). The majority of these GPCRs are from the *Rhodopsin* family but there are a few exceptions,

such as the glucagon receptor, GCGR, smoothened receptor, SMO and the metabotropic glutamate receptor, GRM1 from the *Secretin*, *Frizzled* and *Glutamate* families, respectively.

Table 1.1 GPCRs with their structures resolved by x-ray crystallography or NMR spectroscopy.

Receptor name	Gene	Species	Year	References
Rhodopsin	RHO	Bovine/squid	2000-2013	(4-7)
B_1 Adrenergic receptor	ADRB1	Turkey	2008-2014	(8-12)
B_2 Adrenergic receptor	ADRB2	Human	2007-2014	(13-18)
Adenosine A_{2A}	ADORA2A	Human	2008-2012	(9,19-21)
Chemokine CXCR4	CXCR4	Human	2005	(22)
Dopamine receptor 3	DRD3	Human	2010	(23)
Histamine receptor 1	HRH1	Human	2011	(24)
Muscarinic M2	CHRM2	Human	2012-2013	(25,26)
Muscarinic M3	CHRM3	Human	2012	(27)
Kappa-opioid receptor	OPRK1	Human	2012	(28)
Mu-opioid receptor	OPRM1	Mouse	2012	(29)
Delta-opioid receptor	OPRD1	Human/Mouse	2012-2013	(30,31)
Nociceptin receptor	OPRL1	Human	2012	(32)
Protease-activated (PAR1)	F2R	Human	2012	(33)
Neurotensin receptor 1	NTSR1	Human	2012-2014	(34,35)
Chemokine CXCR1†	CXCR1	Human	2012	(36)
Sphinogosine-1 receptor 1	S1P1	Human	2012	(37)
Glucagon receptor	GCGR	Human	2013	(38)
Smoothened receptor	SMO	Human	2013-2014	(39-41)
5-hydroxytryptamine	HTR2B	Human	2013	(42-44)
Chemokine CCR5	CCR5	Human	2013	(45)
Glutamate receptor	GRM1	Human	2014	(46)
Purinergic $P2Y_{12}$ receptor	P2RY12	Human	2014	(47,48)

† structure resolved by NMR spectroscopy. Coloured backgrounds indicate GCPR family; *Rhodopsin* (blue), *Secretin* (green), *Frizzled* (purple), *Glutamate* (orange).

Despite low sequence homology, all GPCRs share a common structure comprised of 7 transmembrane domains (TMs) that exist as α-helices, 3 extracellular and intracellular loops, an extracellular amino-terminus (N-terminus) and an intracellular carboxy-terminus (C-terminus), shown in Figure 1.2A as a flattened representation. The free protein within the lipid bilayer forms a barrel-like structure, shown in Figure 1.2B, which creates a binding pocket for many ligands, although ligand binding does not exclusively occur at this site.

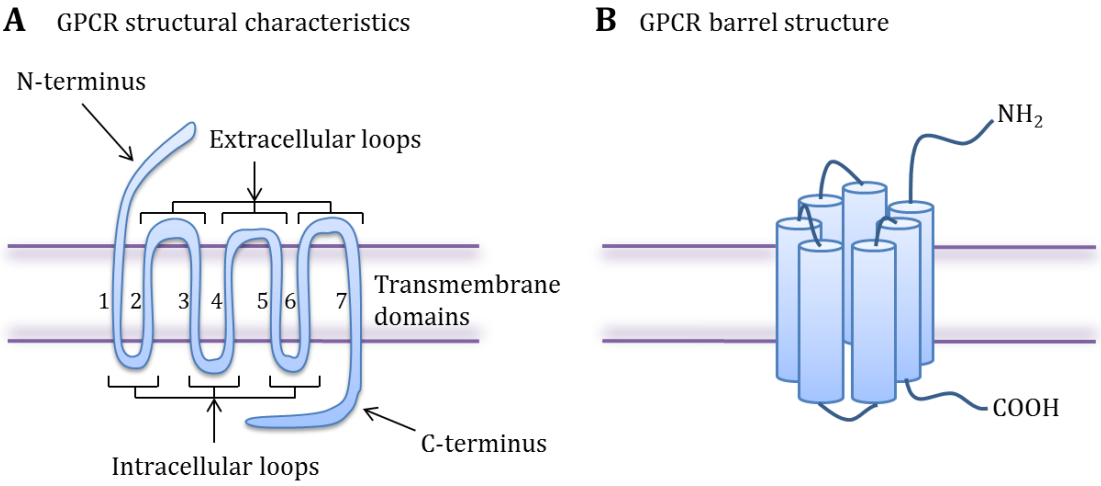


Figure 1.2 Schematic of GPCR structure.

(A) Flattened GPCR schematic to show characteristic structural features; amino-terminus (N-terminus), carboxy-terminus (C-terminus), 7 transmembrane domains, extracellular and intracellular loops. **(B)** Representation of the barrel structural confirmation that GPCRs naturally form in the lipid bilayer. Blue cylinders represent amino acid α-helixes, purple lines represent the lipid bilayer, NH_2 (amino-) and COOH (carboxyl).

Diversity within this common structure, which can occur in all parts of the GPCR structure (TMs, extracellular loops, side chains), allows variations within the size and shape of the GPCR, as well as binding pocket selectivity. These factors lead to a vast array of ligands that can activate these receptors. These ligands include photons, ions such as hydrogen and calcium, peptides, lipids, hormones and proteins. Generally, the sites in which the largest amount of diversity occurs in are within the C-terminus, the intracellular loop between TMs 5 and 6 and the N-terminus (49). The diversity in the C-terminus and the intracellular loops are thought to be almost exclusively related to intracellular signalling events, i.e. binding of the heterotrimeric G-protein. Diversity within the N-terminus differs from family to family and is more important in the *Glutamate* and *Secretin* family where the N-terminal tail can be 350-600 amino acids long, and even longer in the *Adhesion*

family where it is highly glycosylated to form rigid structures (50). Within the *Rhodopsin* family, N-terminal diversity is not as extensive as the other families but will usually occur in the extracellular loops.

In terms of drug discovery, being able to predict the site of ligand binding would greatly improve rational drug design but the extensiveness of GPCR diversity makes this process very difficult. Unfortunately, ligand size alone is not a determining factor and having small molecules or ions as ligands does not necessarily mean that they will activate the receptor within the binding pocket. For example, the glutamate receptor, GRM1, has a "venus flytrap" module that consists of the N-terminal tail forming two lobes that exist in open and closed conformations for binding of its ligand, glutamate (51).

GPCR activation

One of the major paths in GPCR research has been to examine how ligand binding causes intracellular signalling. The current data supports a multi-state mechanism in which specific changes in the GPCR structure are key to the receptor's activation (Figure 1.3). This builds on the original model of an inactive (R) and active (R*) state, introducing intermediary conformations (R' and R'') that are crucial to receptor activation. Rhodopsin, Adenosine A_{2A} and β_2 adrenergic receptor are three *Rhodopsin* family GPCRs that have had crystal structures resolved in a multitude of these conformational states (ligand free, bound by agonist, inverse agonist, antagonist, thermostabilised, nanobody stabilised, Table 1.1). GPCRs are believed to have a dynamic behaviour and certainly in the case of amino acids, movement does occur within the side chains irrespective of activation status (52). This means that proteins can have many structural conformations: both stable and unstable, but it is thought that due to the very nature of crystallisation only the more stable conformations have been resolved, and so there could be many other conformational states not yet identified (53). Surprisingly, even though sequence homology is low between Rhodopsin, Adenosine A_{2A} and the β_2 adrenergic receptor, common features in major conformational changes (i.e. helical shifts) have been identified for receptor activation. For example, the intracellular parts of TM 5 and 6 have been shown to shift in an outward direction in its activated state (9,54). It has also been demonstrated that there are backbone rearrangements in the TM 7, and some specific movements in TM 3. If there are common features in GPCR activation then this could help to explain how a range of ligands and receptors activate similar signalling cascades, but more research is required to fully elucidate such mechanisms.

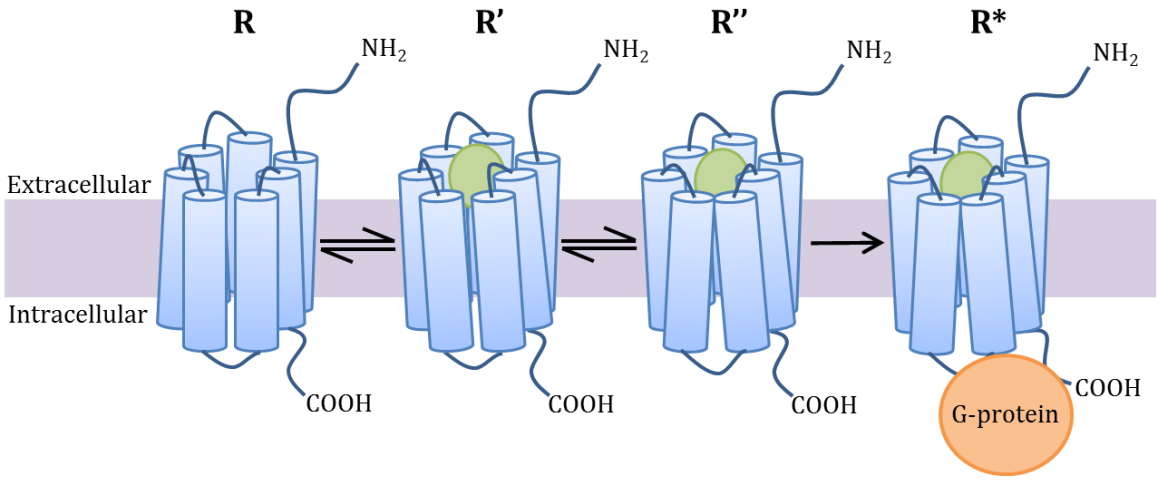


Figure 1.3 Schematic showing GPCR conformational states that lead to activation and signalling.

R represents a GPCR in an inactive conformation, either unbound, bound to an inverse agonist or to an antagonist (not depicted). **R'** represents a GPCR that has bound to its ligand (shown as green oval) with only minor conformational changes. **R''** represents an active GPCR. The ligand is bound and major conformational changes have occurred that allows the heterotrimeric G-protein to bind. **R*** is the GPCR in complex with the G-protein. A full signalling state is when GDP is released and replaced with GTP which allows intracellular signalling to occur. The double arrow signifies a reversible reaction and the single arrow is a one-way reaction.

G-protein-dependent signalling

G-proteins are the most common form of signalling for GPCRs. The G-protein is a heterotrimeric protein consisting of three subunits, α, β and γ (denoted as Gα, Gβ and Gγ, respectively), that are membrane-bound by lipid anchors on the Gα and Gγ subunits. Their name, guanine nucleotide-binding protein, arises from their mode of action. In an inactive state, guanosine diphosphate (GDP) is bound to the active site of the Gα subunit. Once a receptor has been activated and is within its R'' conformational state, G-proteins can bind to the GPCR. Little is known about the interactions between the GPCR and the G-protein. X-ray crystallography of GPCRs bound to their appropriate G-protein is difficult due to instability issues with and without a bound guanine nucleotide (15,55). Crystallisation of the β_2 adrenergic receptor with its G-protein revealed that only Gα interacts directly with the receptor while Gβ and Gγ aid stabilisation of the Gα complex (15). Site-directed mutagenic studies have identified that the third intracellular loop (Figure 1.2) and the C-terminal tail are very critical for binding with the G-protein (56).

It is believed that binding of the G-protein to the receptor causes conformational changes within the Gα subunit (57). This then triggers the release of GDP which leaves an active site that has high affinity for guanosine triphosphate (GTP). When GTP binds to the active site of Gα this also causes conformational changes within the heterotrimeric protein and leads to the dissociation of Gα-GTP from the receptor, and also from Gβ and Gγ, which remain as a heterodimer (Gβγ). Gα-GTP and Gβγ, which are still membrane-bound, can then activate a range of downstream effector molecules. This process continues until the Gα subunit hydrolyses GTP into GDP and then Gα-GDP re-associates with Gβγ. There are 21 different Gα subunits, 6 Gβ and 12 Gγ within humans (58). The Gα subunits are divided into four groups based on amino acid sequence; $G\alpha_q$ $G\alpha_s$ $G\alpha_i$ $G\alpha_{12/13}$ and all activate specific effector molecules. All receptors signal via specific G-proteins and in certain instances they can signal via multiple G-proteins, usually $G\alpha_q$ and $G\alpha_i$.

$G\alpha_q$ signalling is most commonly associated with the activation of phospholipase Cβ (PLC-β) and the influx of intracellular calcium (Figure 1.4). PLC-β is an enzyme that metabolises phospholipids. In this signalling cascade, the membrane-bound phospholipid phosphatidylinositol-4,5-biphosphate (PIP_2) is broken down by PLC-β to form inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) which causes an amplification of signalling as both IP_3 and DAG can activate further signalling cascades (59). DAG will activate a protein kinase enzyme known as protein kinase C (PKC). There are many isozymes of PKC (alpha, beta, delta, epsilon, eta, gamma, lambda, mu, iota, theta and zeta) that are categorised depending on their signalling requirements (60,61). Conventional isozymes (alpha, beta and gamma) are calcium and DAG-dependent. Novel isozymes (delta, epsilon, eta, mu and theta) are independent of calcium but are activated by DAG and phosphatidylserine. Atypical isozymes (lambda and zeta) are thought to be independent of both calcium and DAG. Due to the vast array of PKC isozymes, multiple effector molecules can be activated, such as myosin phosphatase inhibitor, CPI-17, the glycogen synthase kinase 3β (GSK3β) and the kinase c-Raf-1 (62-64). Once again, these can activate multiple pathways which lead to gene transcription and regulation, but most of these have not been fully elucidated. However, c-Raf-1 can activate the mitogen-activated protein kinase (MAPK) pathway, leading to Erk activation and translocation into the nucleus, where it can phosphorylate many transcription factors.

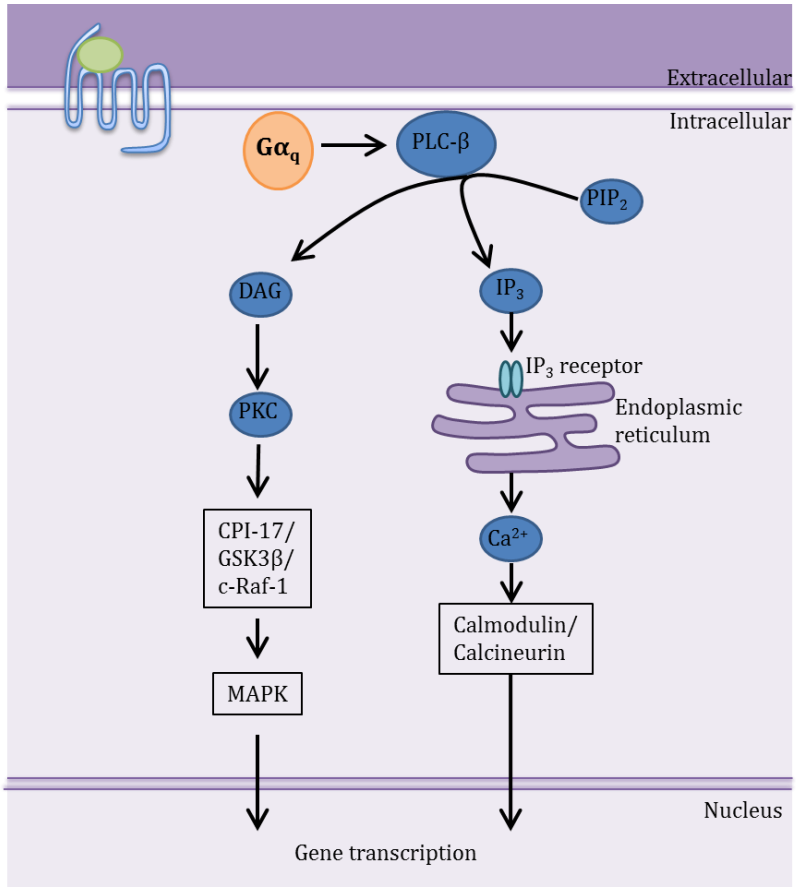


Figure 1.4 Schematic representation of $G\alpha_q$ signalling pathway.

$G\alpha_q$ can activate phospholipase Cβ (PLCβ) which converts phosphatidylinositol-4,5-biphosphate (PIP_2) to diacylgycerol (DAG) and inositol-1,4,5-triphosphate (IP_3). DAG activates protein kinase C (PKC) which can activate multiple pathways; myosin phosphatase inhibitor (CPI-17), glycogen synthase kinase 3β (GSK3β), kinase c-Raf-1 and mitogen-activated protein kinase (MAPK). IP_3 activates the IP_3 receptor which depletes calcium (Ca^{2+}) store which can activate multiple pathways including calmodulin and calcineurin. Black lined boxes indicate possible signalling pathways.

The other pathway involves IP_3 which is a unique inositol phosphate as its target is a channel (Figure 1.4). IP_3R is a channel located on the endoplasmic reticulum and its main function is to regulate the calcium stores (65). On binding of IP_3 to IP_3R, major conformational changes occur within the channel which allow the release of calcium into the cytosol (66). This is a rapid process and fluxes in intracellular calcium can be detected within seconds of GPCR activation. Calcium can affect multiple pathways as well as proteins, such as calmodulin and calcineurin, which can regulate gene transcription.

$G\alpha_s$ signalling is mostly associated with the generation of cyclic adenosine 3,5-monophosphate (cAMP) (Figure 1.5). $G\alpha_s$ can activate membrane-bound adenylyl cyclase

which will then convert cytosolic adenosine triphosphate (ATP) into cAMP. cAMP can then activate exchange proteins activated by cAMP (EPACs) like cAMP-regulated guanine nucleotide exchange factors 1 and 2 (cAMP-GEFI and cAMP-GEFII) and the closely-related PDZ-guanine nucleotide exchange factor 1 (PDZ-GEFI) (67). These eventually lead to the activation of the MAPK pathway. cAMP can also activate the GTP exchange protein, protein kinase A (PKA), which is a tetrameric protein made up of two catalytic subunits. cAMP can bind these two subunits which causes a conformational change leading to the activation of this enzyme. PKA can then activate the cAMP responsive element binding protein (CREB) which is a transcription factor (68). PKA can also inhibit certain pathways such as GSK3 and α-adducin which are thought to be involved with oncogenesis and skeletal rearrangement, respectively (69). $G\alpha_s$ can also activate Src tyrosine kinase family members c-Src and Hck which are thought to be linked with the regulation of G-proteins and GPCRs (70).

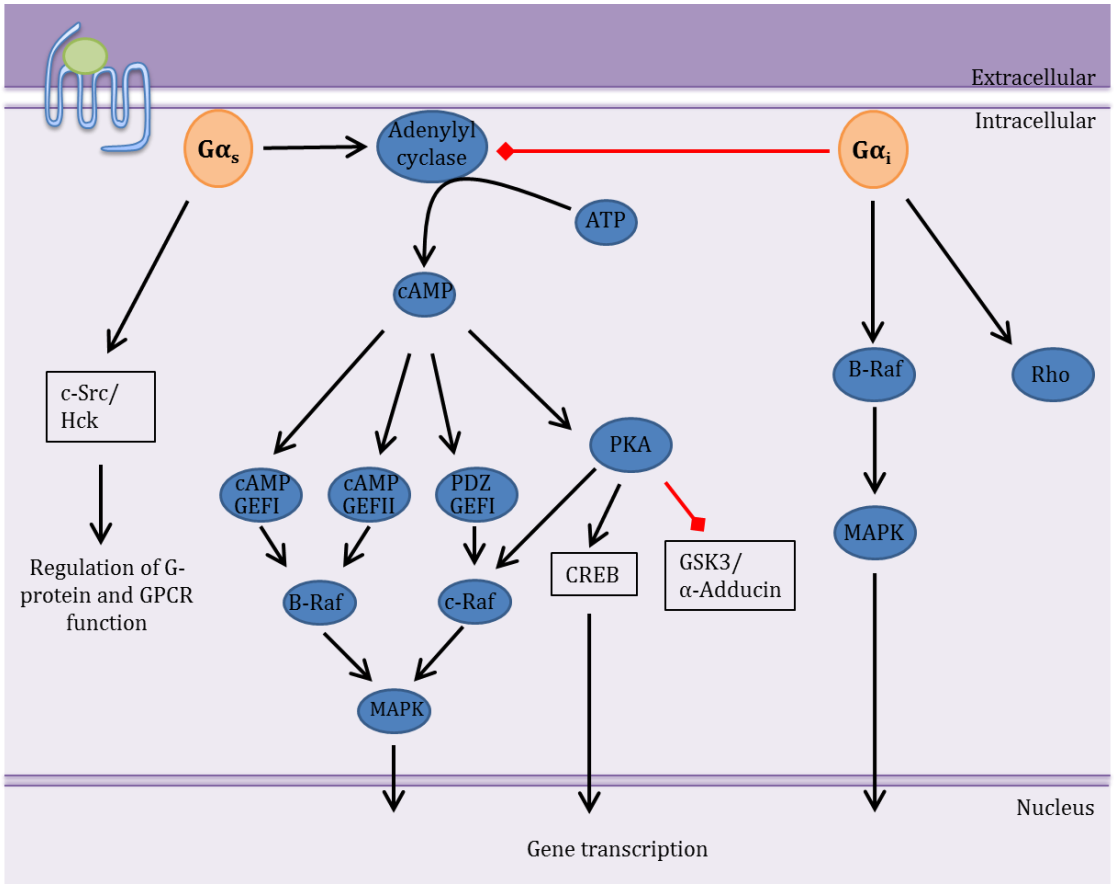


Figure 1.5 Schematic representation of $G\alpha_s$ and $G\alpha_i$ signalling pathways.

$G\alpha_s$ can directly activate Src tyrosine kinase family members c-Src and Hck. It can also activate adenylyl cyclase which converts adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP) thereby activating multiple signalling pathways; guanine exchange factors (GEFs), kinases B-Raf and c-Raf, mitogen-activated protein kinases (MAPK), protein kinase A (PKA). PKA activates cAMP responsive element binding

protein (CREB) and inhibits multiple pathways including glycogen synthase kinase 3β (GSK3β) and α-adducin. $G\alpha_i$ inhibits adenylyl cyclase and can also activate multiple pathways including B-Raf, MAPK and the GTPase Rho. Red diamond ended lines indicate an inhibition of signalling pathway. Black lined boxes indicate possible signalling pathways.

$G\alpha_i$ signalling pathways are commonly associated with the inhibition of cAMP production (Figure 1.5). $G\alpha_i$ will inhibit adenylyl cyclase and therefore reduce cytosolic levels of cAMP. This means that there will be a reduction in PKA activation and so $G\alpha_i$ can be considered a negative regulator. $G\alpha_i$ is also thought to be able to activate B-Raf which will lead to the MAPK signalling pathway and Rho which is thought to be involved with actin cytoskeleton rearrangement (71,72). Identification of this pathway has been refined by the inhibitor pertussis toxin. Pertussis toxin is produced by the bacteria *Bordetella pertussis* and works by inducing ADP-ribosylation of the $G\alpha_i$ subunit. This inhibits the interaction between $G\alpha_i$ and the receptor therefore inhibiting signal transduction (73).

$G\alpha_{12/13}$ is the least well characterised G-protein signalling pathway due to the difficulties in differentiating it from the other signalling pathways. It is most commonly associated with the activation of the Rho GEFs, and activation of the GTPase Rho is often cited as evidence for activation of this pathway (Figure 1.6). It is thought that $G\alpha_{12/13}$ can also activate other pathways which involve Ras (and activation of MAPK pathway), the c-Jun N terminal kinases (JNKs) and directly the MAPK pathway through MEK5/Erk5 all of which lead to various regulations of gene transcription (74-76).

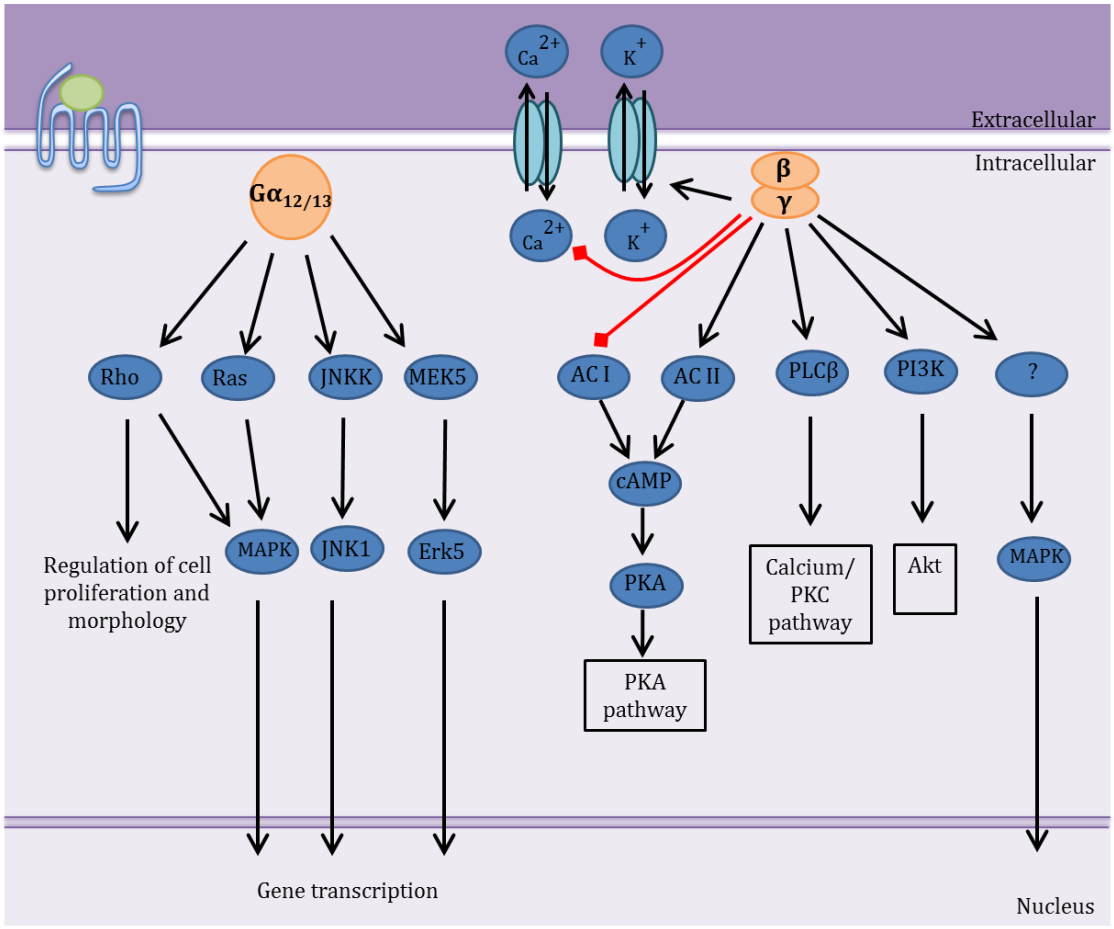


Figure 1.6 Schematic representation of $G\alpha_{12/13}$ and $G\beta\gamma$ signalling pathway.

$G\alpha_{12/13}$ can activate multiple pathways including the GTPase Rho, Ras, c-Jun N-terminal kinases (JNKK and JNK1) and the mitogen-activated protein kinase (MAPK) pathways (this includes MEK5 and Erk5). $G\beta\gamma$ inhibits and activates adenylyl cyclase (AC I and AC II) which produces cyclic adenosine monophosphate (cAMP) that can activate the protein kinase A (PKA) signalling pathway. It can activate the MAPK pathway through unknown (?) upstream effectors and activate phospholipase Cβ (PLCβ) and phosphoinositide 3-kinase (PI3K) pathways. It also inhibits and activates ion channels for calcium (Ca^{2+}) and potassium (K^{+}) respectively. Red diamond ended lines indicate an inhibition of signalling pathway.

The $G\beta\gamma$ heterodimer also has the ability to regulate specific effector molecules. This is a relatively new G-protein signalling pathway compared to the extensive research that has been carried out on $G\alpha$ signalling and its physiological role is still to be elucidated. However the research to date has shown that $G\beta\gamma$ can activate and inhibit certain pathways such as ion channels, adenylyl cyclase and MAPK (although specific upstream effectors remain to be determined). It is also able to activate PLCβ which leads to IP_3 and DAG production therefore depleting calcium stores and activating PKC signalling

pathways. Finally the phosphoinositide 3-kinase (PI3K) can be activated which leads to the protein kinase B (Akt) signalling cascade. It is important to note that all these signalling pathways are not activated at once. It is very dependent on the specific subunit being used and the constitutive expression level of any of the effector molecules within these signalling cascades (76).

GPCR signal termination and G-protein-independent signalling

Arrestins are a small family of proteins that can form complexes with GPCRs for the regulation of receptor function. There are four different arrestins; visual arrestin, cone arrestin, β-arrestin 1 and β-arrestin 2. Visual and cone arrestins are located in the retina as they are involved with light receptor functions, whilst β-arrestins are ubiquitously expressed. After receptor activation and dissociation of the G-protein complex, GPCR kinases (GRKs) will phosphorylate the intracellular domains of the GPCR, which intrinsically reduces receptor activation (a form of desensitisation) (Figure 1.7). β-arrestins can then bind to the intracellular part of the GPCR which blocks any further G-protein binding to the receptor and so aids desensitisation of signal transduction. β-arrestins will then target the GPCR to clathrin-coated pits, where β-arrestin will bind clathrin and the AP-2 adaptor complex, which can bind various elements of clathrin endocytic machinery such as dynamin. The receptor is endocytosised in clathrin-coated vesicles where it can undergo one of two processes. In the first process, the GPCR can be dephosphorylated and dissociate from its ligand, meaning that the receptor can be recycled back to the surface membrane. The second process is degradation by lysosomes.

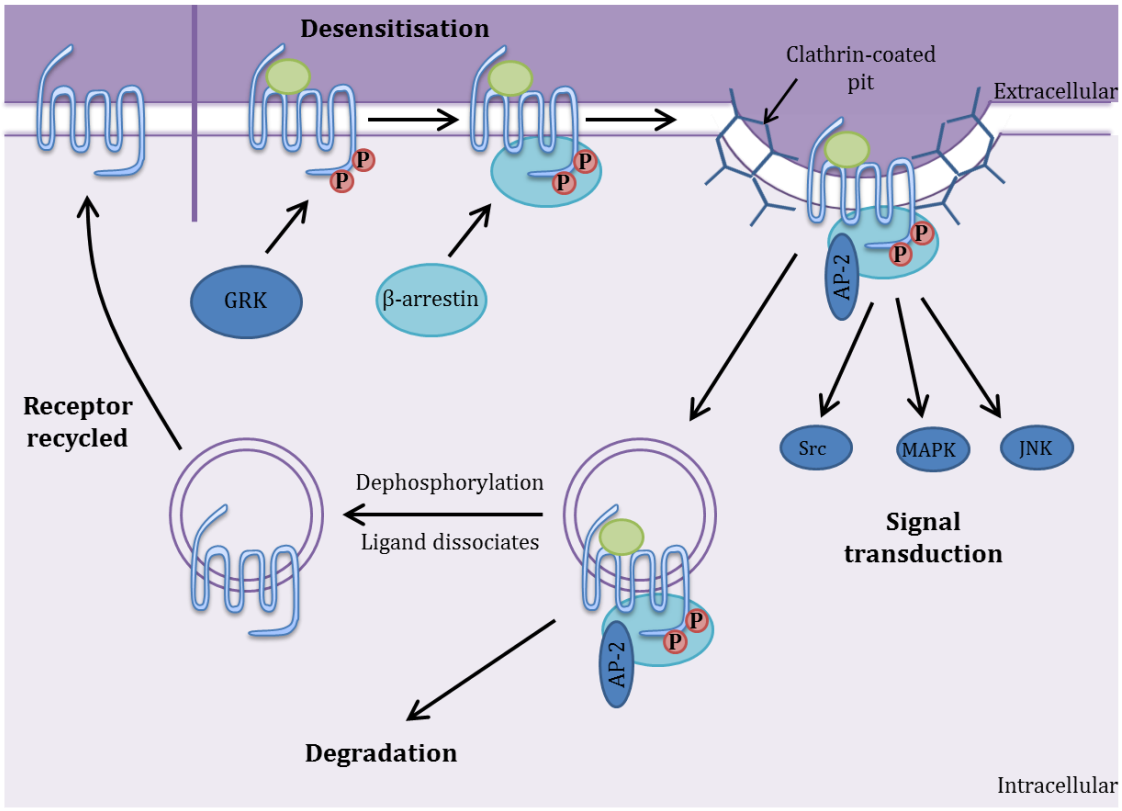


Figure 1.7 Schematic representation of GPCR signal termination and alternative G-protein-independent signalling.

Desensitisation - activated receptors are phosphorylated by GPCR kinases (GRKs). β-arrestin binds the phosphorylated GPCR and targets clathrin coated pits. β-arrestin binds adapter protein 2 (AP-2) and clathrin (blue hexagonal shapes). The GPCR complex is endocytosed into endosomes or lysosomes. **Degradation** – The GPCR complex within the lysosome is degraded. **Recycling** – The GPCR complex within the endosome is dephosphorylated and the ligand dissociates. The GPCR is transported back to the surface membrane. **Signal transduction** - β-arrestin can activate multiple pathways including tyrosin kinase Src, mitogen-activated protein kinases (MAPK) and c-Jun N-terminal kinases (JNK).

β-arrestins may also play a direct role in signalling, independent of G-proteins. Studies have shown that tyrosine kinase Src is able to directly bind β-arrestin (77). Recruitment of Src and other Src family members (Hck and Fgr) have been demonstrated in the rat K-Ras transformed kidney cell line, KNRK, and in human neutrophils towards the neurokinin-1 receptor and CXCR1, respectively (78,79). This is not the only signalling pathway that was identified. In KNRK cells, stimulated protease-activated receptor 2 was found to form complexes with β-arrestin, Raf-1 and activated Erk1/2 (78). Activation of the angiotensin II type 1a receptor has been found to form complexes with other effector molecules, namely β-arrestin, Raf-1, MEK1 and Erk1 (80). β-arrestin has also been shown to act as a

scaffold of JNK by binding of the C- and N-termini respectively (81). The identification of such G-protein-independent GPCR signalling pathways contrasts with the "classical" view of GPCRs that is well established. Continued researched has strengthened this alternative picture and there is now a call to rename GPCRs, seven transmembrane receptors, 7-TMs (82).

1.1.3 Discovery of cysteinyl leukotrienes and their synthesis

Discovery

More than 70 years ago Feldberg and Kellaway demonstrated that contractions of smooth muscle caused by treatment with various snake venoms were not wholly the result of the release of histamine but also the result of the enzymatic actions of venom to form a "slow-reacting smooth muscle-stimulating substance" (83). The contractile actions of this substance on jejunum of sensitised guinea pigs resembled that of anaphylactic antigens, implying for the first time that histamine alone does not cause anaphylaxis, but instead a combination of both histamine and the "slow-reacting smooth muscle-stimulating substance" (84). By the 1960s Feldberg and Kellaway's discovery was renamed the "slow-reacting substance of anaphylaxis" (SRS-A) by Brocklehurst who showed that even in the presence of anti-histamines, SRS-A-induced potent bronchial constriction (85). These actions were shown to be pharmacologically distinct from other smooth muscle constriction causing agents such as bradykinin, 5-hydroxytryptamine (serotonin) and substance P (86).

Elucidating the structure of SRS-A involved several spectroscopic and analytical tools from a multitude of research groups. Initial investigations were hampered by impure, low yield extraction methods but several groups were able to ascertain that SRS-A was a low molecular weight acidic lipid that contained sulphur and carboxylic acids with hydroxyl groups (87,88). Labelling techniques also concluded that the molecule incorporated arachidonic acid (89,90). A few years later, using SRS-A derived from calcium ionophore treated rat mastocytoma cells, the structure of SRS-R was finally identified as not one molecule, but three bioactive molecules (91,92). As these three molecules were structurally related to other previously identified arachidonic acid metabolites derived from leukocytes, leukotriene A_4 (LTA_4) and leukotriene B_4 (LTB_4), and also contained three conjugated double bonds (a "triene"), they were termed leukotriene C_4, D_4 and E_4 (LTC_4, LTD_4 and LTE_4, respectively) (Figure 1.8).

Synthesis

Leukotrienes are synthesised upon activation of many cell types, including mast cells, basophils, eosinophils, monocytes and macrophages. Cell activation initiates leukotriene production by inducing the translocation of $cPLA_2$ to the nuclear membrane in a calcium-dependent manner. $cPLA_2$ can then hydrolyse phospholipids in the nuclear membrane with a specific preference for yielding arachidonic acid (Figure 1.8) (1). Free arachidonic acid then interacts with 5-lipoxygenase (5-LO) and 5-lipoxgenase-activating protein (FLAP). Like $cPLA_2$, 5-lipoxygenase is regulated by calcium mobilisation and will translocate from the cytosol to the nuclear membrane upon cell activation (93). FLAP which is constitutively localised in the nuclear membrane, does not have any enzymatic activity but acts as a scaffold by binding arachidonic acid and enhancing substrate-enzyme interactions (94). Arachidonic acid metabolism by 5-lipoxygenase forms the unstable epoxide known as LTA_4, which continues metabolism by one of two routes. Hydrolysis of LTA_4 by LTA_4 hydrolase (LTA_4H) will convert LTA_4 to LTB_4 within the cytoplasm and potentially within the nucleus (95). The second pathway for LTA_4 metabolism involves the enzymatic conjugation of reduced glutathione by LTC_4 synthase (LTC_4S) at the nuclear membrane. This yields the first of the cysteinyl leukotrienes, LTC_4 (96). Both LTB_4 and LTC_4 are transported by specific chaperone proteins, namely multidrug resistance-associated proteins (MRP4 and MRP1, respectively), to the cell surface where they are exported (97-99). LTB_4 and LTC_4 are not the only molecules that are transported across the cell membrane. It has been suggested that even LTA_4 can be exported to other non-leukocyte cells containing LTA_4 metabolising enzymes. These “acceptor” cells, namely endothelial cells and potentially platelets, have the ability to restore leukotriene levels in the absence of leukotriene producing immune cells (100,101).

In the extracellular space, LTC_4 is in a peptide moiety and so can undergo further metabolism by sequential amino acid hydrolysis. Firstly, LTC_4 reacts with γ-glutamyl transpeptidase (γ-GT) which removes glutamic acid to form LTD_4 (102-104). Next, the glycine residue is removed from LTD_4 by dipeptidase to produce the last of the cysteinyl leukotriene metabolites, LTE_4 (105). As previously discussed, all leukotrienes can activate specific receptors, GPCRs.

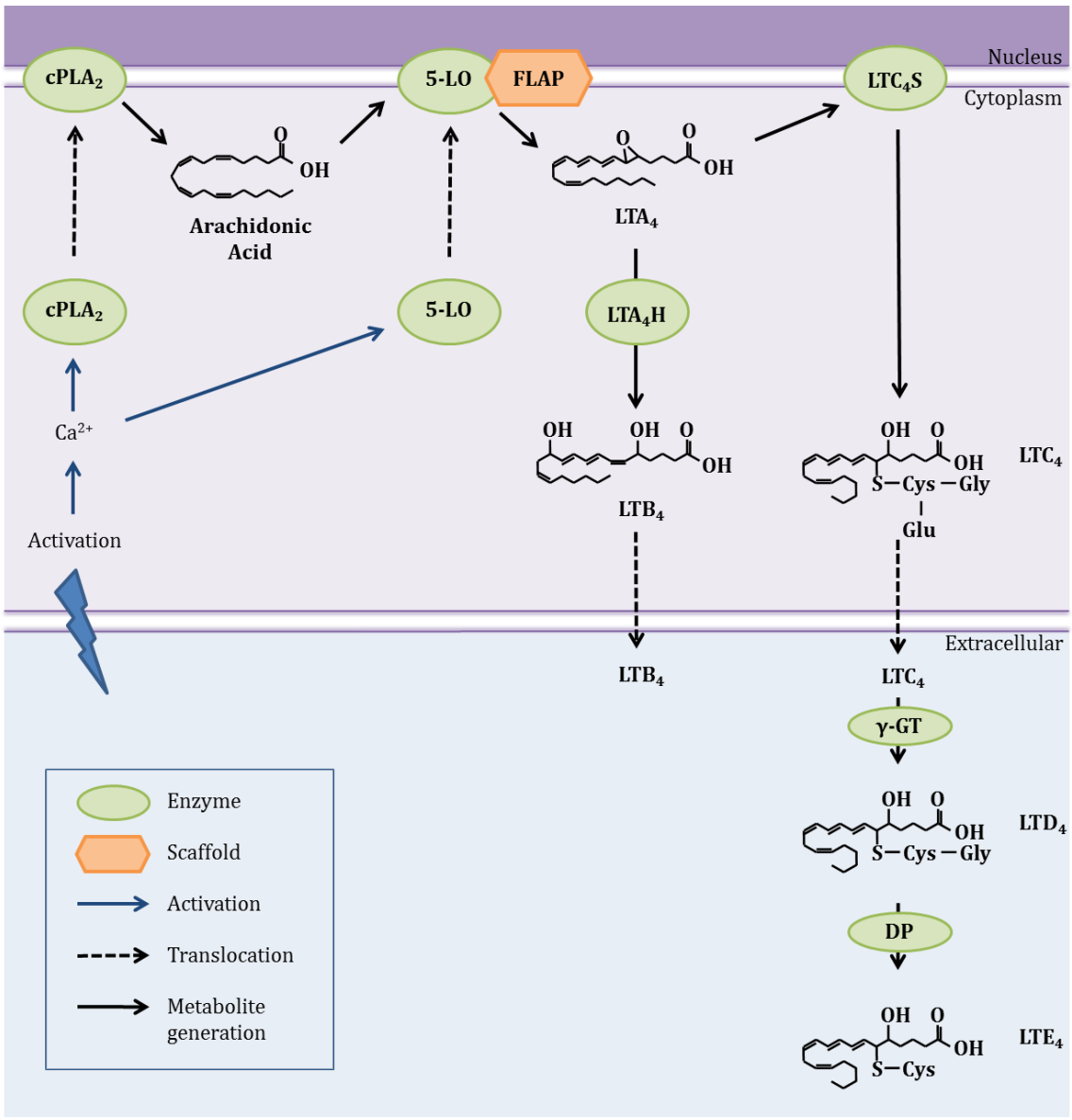


Figure 1.8 Schematic of leukotriene synthesis.

Cytosolic phospholipase A_2 ($cPLA_2$) and 5-lipoxygenase (5-LO) translocate to the nuclear membrane upon cell activation. $cPLA_2$ liberates arachidonic acid from the membrane which reacts with 5-LO and 5-lipoxygenase activating protein (FLAP) to yield leukotriene A_4 (LTA_4). LTA_4 then reacts with either LTA_4 hydrolase (LTA_4H) or LTC_4 synthase (LTC_4H) to produce leukotriene B_4 or C_4 (LTB_4 and LTC_4). LTB_4 and LTC_4 are transported extracellularly where LTC_4 undergoes sequential amino acid hydrolysis by γ-glutamyl transpeptidase (γ-GT) and dipeptidase (DP) to yield leukotriene D_4 and E_4 (LTD_4 and LTE_4).

1.1.4 Cysteinyl leukotriene receptors

Despite the identification of the structure of cysteinyl leukotrienes in 1979, cloning of specific cysteinyl leukotriene receptors proved difficult and it took a further two decades before the first receptor was reported. In fact, receptor antagonists such as Montelukast (Singulair®), Pranlukast (Onon®, Azlaire®), Zafirlukast (Accolate®), MK-571 and BAY-U9773 were all developed prior to the cloning of any cysteinyl leukotriene receptor (106-110). The pharmacological activity of these antagonists led a committee appointed by the International Union of Pharmacology (IUPHAR) to characterise two classes of cysteinyl leukotriene receptors. The first was sensitive to the inhibitory actions of the so-called "classical" antagonists (Montelukast, Pranlukast, Zafirlukast and MK-571) and hence denoted the nomenclature $CysLT_1$. The second class was insensitive to these classical antagonists and was termed $CysLT_2$. BAY-U9773 was the only antagonist that could inhibit the actions of both these classes of receptor.

Early pharmacological data showed that Gpp(NH)p, a stable analogue of GTP, was able to alter LTD_4 ligand binding. Modification of such binding was also seen with divalent cations and sodium ions (enhancement and inhibition respectively) strongly suggesting that cysteinyl leukotriene responses were mediated by GPCRs (111,112). In 1999, using reverse pharmacological approaches, i.e. using the receptor to identify the ligand instead of the ligand to identify the receptor, these observations were confirmed. Two independent groups identified the orphan GPCR, HG55 (also termed HMTMF81), as a receptor for cysteinyl leukotrienes (113,114). The receptor's responses were sensitive to the classical antagonists and thus the receptor was renamed $CysLT_1$. A year later 3 independent groups identified the orphan GPCR, HG57 (also termed PSEC0146) as a second cysteinyl leukotriene receptor that was insensitive to the classical antagonists and it was named $CysLT_2$ (115-117).

$CysLT_1$ and $CysLT_2$ are part of the *Rhodopsin* family of GPCRs and more specifically, part of the purine receptor cluster (on the δ branch). $CysLT_1$ shares 38 % homology with $CysLT_2$ based on amino acid structure and they are highly conserved when compared to the mouse versions (88 % and 73 % amino acid homology respectively). Cysteinyl leukotriene receptors share a higher homology with the purinergic receptors (P2Y family) than the other leukotriene receptors BLT_1 and BLT_2 (LTB_4 receptors reside on the γ branch).

$CysLT_1$ characterisation and expression

The human $CysLT_1$ gene, now known as CYSLTR1, is located on the long arm of the X chromosome at position Xq13.2-q21.1 (Table 1.2). The major transcript contains three exons in which the last exon contains the open reading frame which encodes a 337 amino acid structure (118).

Table 1.2 Characteristics of known and potential cysteinyl leukotriene receptors.

	$CysLT_1$	**$CysLT_2$**	**$P2Y_{12}$**	**OXGR1**
Gene Name				
Hs	CYSLTR1	CYSLTR2	P2RY12	OXGR1
Mm	Cysltr1	Cysltr2	P2ry12	Oxgr1
Chromosome				
Hs	Xq13.2-q21.1	13q14.2	3q24-q25	13q32.1
Mm	X D	14 D3	3 D	14 E4
NCBI seq. no.				
Hs	NP_006630	NP_065110	NP_073625	NP_543008
Mm	NP_067451	NP_598481	NP_081847	NP_001001490
Homology				
Hs vs. *Mm*	88 %	73 %	89 %	85 %
Hs-R vs. *Hs*-$CysLT_1$	-	38 %	26 %	28 %
Agonists	LTD_4>C_4>>E_4	LTC_4=D_4>>E_4	ADP, LTE_4?	α-ketoglutarate, LTE_4?
Antagonists	MK-571, Montelukast, Zafirlukast, Pranlukast	HAMI 3379	Clopidogrel	

Hs, Homo sapiens; *Mm*, Mus muscularis; seq., sequence; no., number; vs., versus; R, receptor. Information obtained from NCBI and IUPHAR databases (119). Homologies were calculated using NCBI BLAST Align (120). $P2Y_{12}$ and OXGR1 will be discussed in Chapter 3.

Northern blot analysis of CYSLTR1 expression has indicated that receptor expression is highest in peripheral blood leukocytes and in the spleen (113,114). To a lesser extent, expression has also been detected in the lungs, placenta, gastrointestinal system, heart, pancreas and prostate. There is little or no expression of $CysLT_1$ in the liver, kidney, skeletal muscle or brain. Functional $CysLT_1$ expression has been reported in many cell types and has been summarised in Table 1.3.

Table 1.3 Cell types functionally expressing human $CysLT_1$.

Cell Type	References
Eosinophils	(121,122)
Basophils	(122)
Monocytes/macrophages	(122,123)
Dendritic cells	(124)
$CD34^+$ hematopoietic progenitors	(122)
Mast cells	(125)
B lymphocytes	(122)
Lung fibroblasts	(126)
Astrocytes	(127)
Vascular smooth muscle	(128)
Intestinal epithelial cells	(129)
Th2 cells	(130)
Lung type 2 innate lymphoid cells	(131)

References indicated are initial papers showing functional expression.

$CysLT_1$ is a receptor for all three cysteinyl leukotrienes, LTC_4, LTD_4 and LTE_4 but characterisation of their responses has revealed varying affinities. Analysis of intracellular calcium mobilisation in transfected HEK293 cells showed EC_{50}s of 24 nM, 2.5 nM and 240 nM for LTC_4, LTD_4 and LTE_4 respectively and similar affinities were observed in transfected Cos-7 cells, showing that the rank order of ligands for $CysLT_1$ to be LTD_4>LTC_4>LTE_4 (113,114). This order of ligand affinities has also been observed when pigment dispersion was analysed in $CysLT_1$ transfected melanophores, EC_{50} 0.4 nM, 21 nM and 212 nM (LTD_4, LTC_4 and LTE_4 respectively) (113). LTC_4 and LTD_4 were able to produce similar maximal responses while responses to LTE_4 were shown to be about 40 % of the maximum (114). This has led to the suggestion that LTE_4 works as a "partial" agonist (i.e. unable to reach maximum response of LTC_4 and LTD_4), unlike LTC_4 and LTD_4 which are "full" agonists, this is in agreement with early work on human pulmonary vein preparations (132).

As already noted, $CysLT_1$ induces intracellular calcium mobilisation. This is typically a second messenger for $G\alpha_q$-related signalling, and in the initial characterisation studies by Lynch *et al.* and Sarau *et al.* it was shown that calcium mobilisation in transfected human embryonic kidney 293 (HEK293) cells and cRNA injected oocytes were unaffected by pretreatment of pertussis toxin, a specific inhibitor of $G\alpha_i$ (pertussis toxin mode of action described in section G-protein-dependent *signalling*), suggesting that $CysLT_1$ couples to $G\alpha_q$ only. However, further studies using several different cell types have observed

$CysLT_1$-dependent responses being sensitive to pertussis toxin treatment. For example, in a recent study showing that human Th2 cells selectively express $CysLT_1$, LTD_4-induced intracellular calcium mobilisation was partially inhibited by about 50 % with pertussis toxin treatment. The involvement of $G\alpha_i$ coupling was confirmed by LTD_4's ability to also inhibit forskolin-induced cAMP (130). These observations have also been confirmed in the human monocytic cell lines, THP-1 and dU937, where LTD_4-induced calcium mobilisation was partially inhibited by pertussis toxin, suggesting in certain cell systems $CysLT_1$ can couple to both $G\alpha_q$ and $G\alpha_i$ (133). It has also been observed in human elutriated monocytes that pertussis toxin can completely inhibit LTD_4-induced calcium mobilisation, gene expression and chemotaxis suggesting a $G\alpha_i$ only coupled $CysLT_1$ (134). Such differences in observed responses could be due to constitutive expression levels of these G-proteins differing from cell type to cell type. Downstream signalling will be discussed further in Chapter 6.

$CysLT_2$ characterisation and expression

The $CysLT_2$ receptor gene, also known as CYSLTR2, is located on chromosome 13q14.2 (Table 1.2). There are six exons within the CYSLTR2 gene in which exons 1-5 are located in the untranslated region and exon 6 contains the full coding region with no introns. This encodes a protein of 346 amino acids long. Eight transcripts have been identified in which exons 1 and 6 are always present and expression seems to be cell type dependent (135). $CysLT_2$ shares only 38 % homology with $CysLT_1$ and 73 % homology with the mouse version when comparing amino acid sequences (Table 1.2).

Northern blot analysis has identified a very distinct pattern of CYSLTR2 expression from CYSLTR1 with only few exceptions. The most striking differences are within the heart and the central nervous system (115-117). *In situ* hybridisation revealed Purkinje fibre cells, monocytes and fibroblasts derived from the brain, atrium and adrenal glands to be expressing CYSLTR2. Extensive northern blot analysis has revealed high CYSLTR2 expression throughout the central nervous system, including medulla, mid-brain, hypothalamus, amygdala, pituitary, putamen and the frontal cortex. The hippocampus showed no CYSLTR2 expression. Such extensive expression within the brain and heart is in stark contrast with CYSLTR1, although immunohistochemistry analysis of brain tissue has found that $CysLT_1$ expression level is increased in neuron- and glial-appearing cells around the site of injury or tumour (136). Moderate expression has also been seen in the spleen and peripheral blood leukocytes (115). Unfortunately due to the previous lack of any selective $CysLT_2$ antagonists (HAMI 3379 the selective $CysLT_2$ antagonist was first

characterised in 2010 (137)), cells functionally expressing $CysLT_2$ are poorly defined (Table 1.4).

Table 1.4 Cell types functionally expressing human $CysLT_2$.

Cell Type	Reference
Coronary artery smooth muscle cells	(138)
HUVECs	(135,139)
Mast cells	(140)

Human umbilical vein endothelial cells (HUVEC). References indicated are initial papers showing functional expression.

The initial papers on $CysLT_2$ by Heise *et al.*, Nothacker *et al.* and Takasaki *et al.*, analysed the second messenger signalling cue, calcium, to characterise receptor responses. All three cysteinyl leukotrienes induced intracellular calcium mobilisation in transfected HEK293 cells with varying affinities (summarised in Table 1.5). All studies concluded that the rank order of ligand affinities were LTC_4=LTD_4>>LTE_4 with LTC_4 and LTD_4 acting as full agonists and LTE_4 as a partial agonist (115-117). LTE_4's partial agonist properties were also observed in human coronary smooth muscle cells (138).

Table 1.5 EC_{50} for cysteinyl leukotriene induced intracellular calcium mobilisation in HEK293 cell transfected with $CysLT_2$.

Study	LTC_4 EC_{50}	LTD_4 EC_{50}	LTE_4 EC_{50}
Heise *et al.* (115)	67 nM	104 nM	2300 nM
Nothacker *et al.* (116)	8.9 nM	4.4 nM	239 nM
Takasaki *et al.* (117)	2.31 nM	2.53 nM	76.8 nM

As $CysLT_2$ also induces intracellular calcium mobilisation, similar to $CysLT_1$, it was important to ascertain whether this was through coupling to $G\alpha_q$, $G\alpha_i$ or both. LTC_4 and LTD_4-induced intracellular calcium mobilisation in cRNA injected *Xenopus Laevis* oocytes were not significantly affected by pertussis toxin treatment (115). In human umbilical vein endothelial cells (HUVECs) LTC_4-induced intracellular calcium mobilisation and early growth response protein-3 (Egr-3) mRNA upregulation was not affected by pertussis toxin treatment. This suggests that $CysLT_2$ couples to $G\alpha_q$ only (135,139). Unfortunately, in

human mast cells LTC_4-induced IL-8 secretion was completely abrogated by pertussis toxin treatment, suggesting that $CysLT_2$ could also couple to $G\alpha_i$, and the ability of $CysLT_2$ to couple to $G\alpha_q$, $G\alpha_i$ or both may be cell-type specific, as for $CysLT_1$ (140).

$CysLT_2$ is a poorly characterised receptor and thus analysis of specific signalling pathways is limited. In HUVEC cells, LTC_4-induced Egr mRNA expression was partially inhibited by the PLCβ inhibitor, U73122, and was fully inhibited by the IP_3 inhibitor, 2APB, but not by pertussis toxin, suggesting that $CysLT_2$ regulates Egr via a $G\alpha_q$, PLCβ, IP_3 signalling pathway (135). In a HEK293 cell line stably expressing $CysLT_2$, LTC_4 induced the phosphorylation of c-Jun and JNK, but the specific JNK inhibitor SP600125 curiously was unable to affect either observation (141). Within this cell type PKC epsilon dominant negative constructs inhibited p65 phosphorylation which could be involved in the activation of NF-κB signalling pathways (141). Egr-3 and -4 has been shown to interact with NF-κB p50 and p65 and so there could be some overlap between these two studies (142). Unfortunately, more research needs to be carried out to fully elucidate $CysLT_2$ signalling pathways.

Cysteinyl leukotriene receptor immunoregulation

$CysLT_1$

The Th2 and mast cell cytokine IL-5 was the first mediator shown to modulate $CysLT_1$ expression and function. In a myeloid leukaemia cell line (HL-60) differentiated into eosinophils, IL-5 was able to upregulate $CysLT_1$ expression level, confirmed at an mRNA and protein level using flow cytometry (121). This increase in CYSLTR1 mRNA was inhibited by the antibiotic actinomycin D (by blocking new RNA synthesis) which suggests that IL-5's regulation of $CysLT_1$ occurs through a transcriptional mechanism. Upregulation of $CysLT_1$ by IL-5 at a protein level has also been seen in peripheral blood isolated eosinophils, although increases at an mRNA level were not statistically significant (143). In the differentiated HL-60 cell line, LTD_4-induced intracellular calcium mobilisation and chemotaxis increased in cells that had been pretreated with IL-5 (121). These responses were fully inhibited by the selective $CysLT_1$ antagonist MK-571 proving that these responses were specifically attributed to $CysLT_1$.

IL-4, another cytokine commonly associated with Th2 cells, also released by basophils, mast cells and eosinophils, is another molecule linked to $CysLT_1$ regulation. In monocytes and monocyte-derived macrophages, IL-4 treatment was able to upregulate $CysLT_1$ expression in a concentration and time-dependent manner, confirmed at both an mRNA

and protein level (118,123,143,144). This has also been observed in eosinophils, T cells and B cells (143). The functional consequence of receptor upregulation has been confirmed using various assays. LTD_4-induced calcium mobilisation was increased with IL-4 pretreatment in monocytes and T cells (123,143). Chemotaxis assays have shown that the weakly chemotactic behaviour of monocytes towards LTD_4 can be augmented with IL-4. LTC_4 and LTD_4-induced CCL2 production by the monocytic cell line THP-1 can also be increased several fold by IL-4 pretreatment (118,123). All functional assays showed that the LTD_4-induced responses were completely inhibited by MK-571. In bronchial smooth muscle cells, IL-4 was unable to increase $CysLT_1$ surface expression but increased LTD_4-induced cell proliferation.

IL-4 has also been implicated in the "priming" of mast cells. Although there were no detectable changes in $CysLT_1$ expression and only modest $CysLT_2$ surface expression changes, IL-4 pretreatment of cultured human mast cells induced the release of TNF-α, IL-5 and CCL4 in response to LTC_4 and LTD_4 (125,140,145). These responses were inhibited by MK-571 suggesting the involvement of $CysLT_1$ or "$CysLT_1$ like receptor". STAT6 has been shown to play an essential role in IL-4 signalling (146,147). The $CysLT_1$ gene promoter region has two STAT6 binding sites which could help to explain IL-4's activity.

In B cells, IL-4 alone is unable to affect $CysLT_1$ expression but co-stimulation with CD40 (crucial for B cell activation) or with irradiated CD154-transfected murine fibroblasts (mimics *in vivo* environment and stimulation within the germinal centre), upregulates $CysLT_1$ expression, confirmed by mRNA and flow cytometry (148). This co-stimulatory effect is also seen with IL-13 instead of IL-4. LTD_4-induced intracellular calcium mobilisation is enhanced by this co-stimulation of CD40 and IL-4 and is completely inhibited by Montelukast.

IL-13, another Th2 cytokine, has also been shown to upregulate $CysLT_1$ at both mRNA and protein levels in monocytes, monocytes-derived macrophages, human foetal lung fibroblasts (HFL-1), bronchial smooth muscle cells and eosinophils, but had no effect on T cells or B cells (123,126,143,144,149). In monocytes these observations were functionally confirmed with intracellular calcium mobilisation and chemotaxis assays (123). IL-13 can also signal through STAT6 and so may work in a similar mechanism to IL-4.

In human airway smooth muscle, LTD_4-induced cell stiffness was enhanced by pretreatment with interferon-γ (IFN-γ), a cytokine secreted by Th1 cells, although IFN-γ was shown to have no effect on $CysLT_1$ mRNA levels in monocytes and monocyte-derived macrophages (123,150).

Not all cytokines upregulate $CysLT_1$ expression. IL-10 can downregulate $CysLT_1$ and $CysLT_2$ mRNA in monocytes and monocyte-derived dendritic cells (DCs). Functional assays observing intracellular calcium mobilisation, FosB and Egr-2 gene expression and chemotaxis showed an inhibition of LTD_4-induced responses in cells that had been pretreated with IL-10 that was not too dissimilar from that of MK-571. This shows that $CysLT_1$ is a highly-regulated receptor affected extensively by a plethora of Th2 cytokines and some Th1 cytokines. This could have huge implications when studying diseases such as asthma, where both cysteinyl leukotrienes and Th2 cell associated cytokines are thought to play critical roles in many of the key pathogenic pathways.

$CysLT_2$

Information on the immunoregulation of $CysLT_2$ is limited, mainly due to the very few cell types that are thought to functionally express $CysLT_2$ only, and the lack of $CysLT_2$ selective antagonists. In HUVECs, cultured primary endothelial cells and eosinophils, IFN-γ has been shown to upregulate $CysLT_2$ mRNA in a dose-dependent fashion while $CysLT_1$ mRNA levels were unaffected (135,151). In HUVECs, LTC_4 and LTD_4-induced calcium mobilisation and LTC_4-induced Egr and COX-2 gene expression was enhanced by pretreatment with IFN-γ (135). These responses were not inhibited by MK-571 suggesting $CysLT_2$ receptor involvement.

IL-1β and TNFα have been shown to decrease $CysLT_2$ mRNA in HUVECs but contrasting studies have shown that these cytokines, as well as TGF-β1, have no effect on receptor mRNA level (152,153).

The fact that both $CysLT_1$ and $CysLT_2$ expression levels are so readily affected by other mediators highlights the importance of considering immunoregulation, especially when analysing cysteinyl leukotriene responses in primary cultured cells. These may not reflect the "true" responses that are seen *in vivo* since receptor expression level may be altered.

1.1.5 Cysteinyl leukotrienes in asthma

Asthma is a chronic respiratory disease that presents as variable airway obstruction associated with airway hyperresponsiveness and inflammation in response to various stimuli. The pathogenesis of asthma involves many different mediators, cell types and genetic variations that unfortunately vary from asthmatic to asthmatic and the exact cause remains poorly determined. Cysteinyl leukotrienes are produced by an array of different

cells (Figure 1.9) and have been shown to be important in many of the key phenotypes that define asthma. These include vascular permeability, increased airway smooth muscle proliferation and contraction, acting as chemotactic agents for the recruitment of inflammatory cells, increased mucus secretion and decrease in mobility, and finally increase of airway hyperresponsiveness (Figure 1.9).

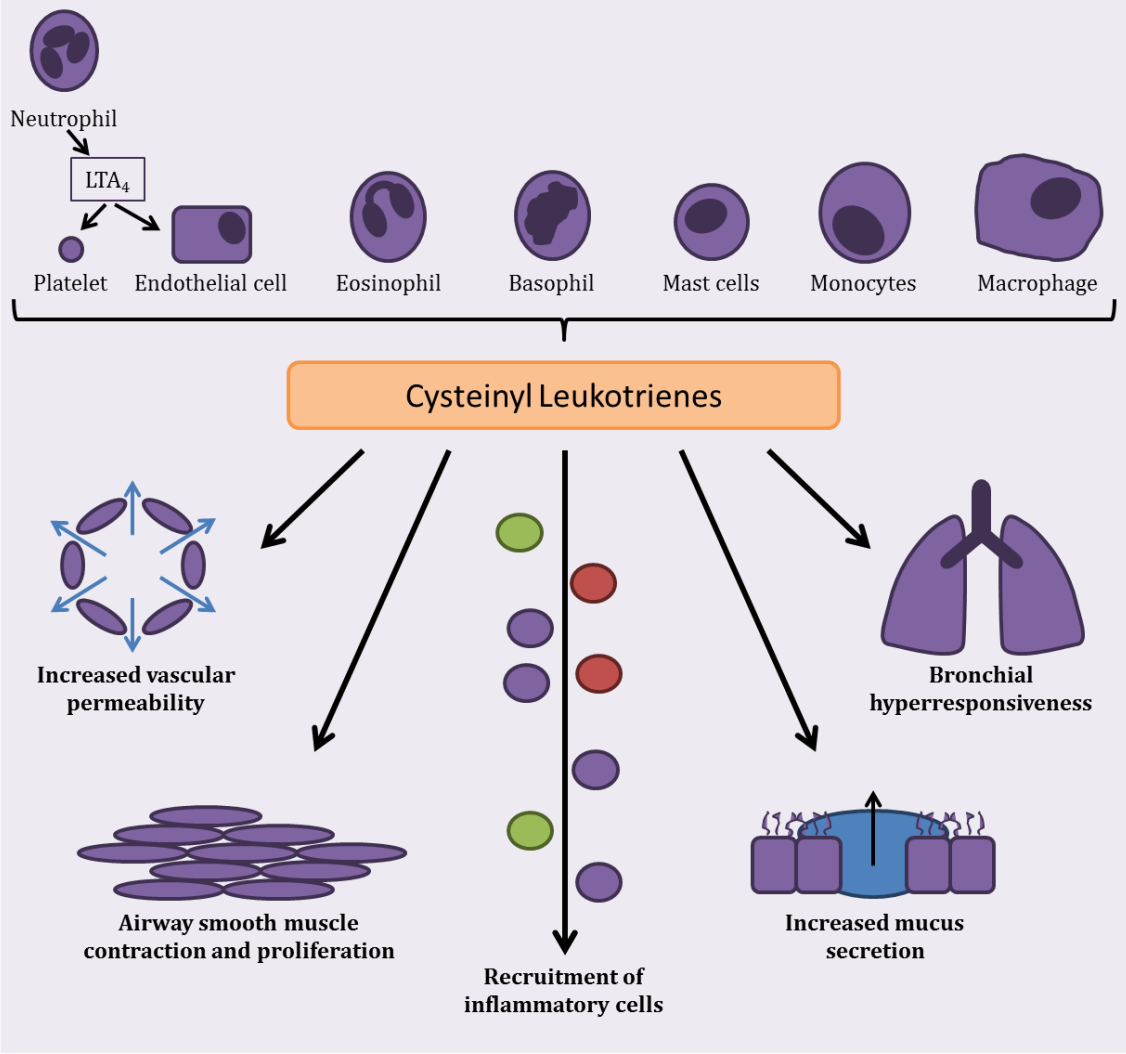


Figure 1.9 Schematic representation of the role of cysteinyl leukotrienes in asthma. Neutrophils, eosinophils, basophils, mast cells, monocytes, macrophages and by the transcellular metabolism of leukotriene A_4 (LTA_4), platelets and endothelial cells can produce cysteinyl leukotrienes. Cysteinyl leukotrienes can induce increased vascular permeability, airway smooth muscle contraction and proliferation, recruitment of inflammatory cells, increase of mucus secretion and bronchial hyperresponsiveness.

Increased vascular permeability

Plasma leakage and mucosal oedema are features of many inflammatory diseases, including asthma. It precedes airway inflammation and exacerbates bronchial constriction by causing oedema of the airway wall and narrowing of the airway lumen (154). Cysteinyl leukotrienes were first implicated in vascular permeability when partially purified rat SRS-A was shown to be able to augment vascular permeability when it was injected into the skin of guinea pigs (155). The three individual components of SRS-A, LTC_4, LTD_4 and LTE_4 were then individually shown to induce leakage of the microvasculature within *in situ* guinea pig tracheal preparation, quantified by extravascular Evans blue dye (156). These responses were not inhibited by indomethacin, an inhibitor of cyclooxygenase, or mepyramine, a histamine H_1 receptor antagonist, suggesting neither cyclooxygenase metabolites nor histamine were indirectly involved (156,157).

In $LTC_4S^{-/-}$ knockout mice (LTC_4S is a key enzyme in cysteinyl leukotriene production), vascular permeability was significantly reduced after antigen challenge (158). In $CysLT_1^{-/-}$ and $CysLT_2^{-/-}$ knockout mice, reduced vascular permeability was also observed after antigen challenge. Although the exact mechanism is not fully understood, these studies further highlight the importance of cysteinyl leukotriene signalling pathway in the induction of vascular permeability (159).

Airway smooth muscle contraction and proliferation

Alterations in airway smooth muscle (ASM) function by hyperplasia, hypertrophy and possibly increased contractility are thought to contribute to asthma pathogenesis. Cysteinyl leukotrienes are potent bronchial constrictors and it has been shown *in vivo* that C_4 and D_4 are ~1000 times more potent than histamine at causing bronchial constriction in healthy individuals (160). Unfortunately, the direct mechanism of cysteinyl leukotrienes effect on airway smooth muscle has yet to be elucidated and responses in ASM cells do not entirely reflect that of the response observed *in vivo*.

The first study implicating cysteinyl leukotrienes in ASM proliferation by Noveral *et al.* analysed the mechanism of thromboxane-induced ASM proliferation in cultured rabbit ASM (161). This study revealed that the mitogenic responses of thromboxane could be abrogated by inhibitors of PLA_2, 5-lipoxygenase and cysteinyl leukotriene receptor antagonists. Thromboxane's actions were shown to induce the release of endogenous cysteinyl leukotrienes, in which LTD_4 was able to induce proliferation of the rabbit ASM (161). These results were supported by a study that used OVA-sensitised Brown Norway

rats to analyse the effect of allergen challenge on ASM mass (162). Repeated OVA challenge increased ASM mass in the large airways of these rats compared to saline. This OVA-induced increase was partially inhibited by MK-571, suggesting that ASM proliferation (hyperplasia) is partly mediated via a MK-571 sensitive receptor (162). Accomazzo *et al.* also showed that strips of human bronchus are able to exhibit contraction to a similar level as histamine but LTD_4 was a far more potent agonist. However, when analysing ASM cultured from the same organ, LTD_4 was significantly less effective at inducing intracellular calcium mobilisation than histamine (the phosphorylation of myosin light chain by calcium is a major pathway in ASM contraction) (163). This adds to a growing body of evidence that LTD_4 alone is unable to alter ASM function in cultured cells. In human cultured ASM, LTD_4 could only increase proliferation in the presence of the growth factor epidermal growth factor (EGF) (164). Similar responses have been seen in rabbit tracheal ASM with insulin growth factor-1 (IGF-1) and human ASM with IL-13 and TGF-β (149,165).

This could suggest that immunoregulation is playing a large role in cysteinyl leukotriene responses and the reason why *in vivo* observations do not quite match with *in vitro* may be due to regulation of leukotriene receptor expression. This type of immunoregulation has already been seen in human cultured ASM by IFN-γ, which was able to enhance LTD_4-induced ASM cell stiffness. Cultured ASM would be devoid of these growth factors and cytokines that would be naturally present at a tissue level.

Recruitment of inflammatory cells

Increasing emphasis has been put on respiratory inflammation in asthma, especially with the use of corticosteroids (used to relieve inflammation) in the treatment of this disease. Inflammation can occur in acute phases, usually associated with asthma attacks, where severe narrowing of the airways (and increased mucus secretion) leads to difficulties in breathing and chronic inflammation. This is associated with persistent inflammation of the airways which increases its sensitivity to a variety of mediators. Cysteinyl leukotrienes have been shown to contribute to inflammation through the recruitment of specific cell subsets both *in vivo* and *in vitro*.

OVA-sensitised guinea pigs have been shown to have an increase in eosinophils in the airway mucosa after OVA challenge. This OVA-induced eosinophilia was inhibited by MK-571 but was not affected by the histamine H_1 and H_2 antagonists, mepyramine and cimetidine or indomethacin (166). In cynomolgus monkeys, the cysteinyl leukotriene receptor antagonist, IC1 198615, was able to inhibit both eosinophil and peripheral blood

lymphocyte infiltration into the bronchial alveolar lavage fluid (BAL) upon antigen challenge (167). Finally in OVA-sensitised C57B1/6 mice, MK-571 was able to significantly inhibit total cell number within the BAL which specifically included the infiltration of eosinophils, CD8 and B cells (168). As all of these responses are sensitive to cysteinyl leukotriene receptor antagonists, this directly associates cysteinyl leukotrienes with inflammatory cell recruitment and is further supported by studies showing that inhalation of cysteinyl leukotrienes (LTC_4, LTD_4 and LTE_4) can induce an increase in eosinophils in BAL and airway mucosa shown *in vivo* in animal models and in human studies (169-172).

Data suggests that cysteinyl leukotrienes are inducing inflammatory cell recruitment via both direct and indirect mechanisms. In the direct mechanism, cysteinyl leukotrienes are acting as chemotactic agents. This has been shown for LTD_4 with isolated human eosinophils and Th2 cells using a Boyden chamber to quantify migration (173-175). The indirect mechanism involves "priming" of these cells. It has also been observed that all cysteinyl leukotrienes can upregulate the adhesion molecule, macrophage adhesion ligand-1 (Mac-1), a process which is inhibited by Montelukast (174). Finally, LTD_4 (LTC_4 and LTE_4 were not tested) can increase eosinophil adhesion which can be blocked by an anti-β_2 integrin antibody and also by Pranlukast (175). This suggests that cysteinyl leukotrienes are not only directly involved in cell migration but can also affect the regulation of eosinophil adhesion molecules which are crucial elements in tissue infiltration.

Increased mucus secretion

Pathological analysis of individuals that have died from acute asthma attacks revealed hyperinflation caused by mucus plugs in the airways (176). These mucus plugs are thought to be formed in severe asthmatics due to enhanced mucus secretion and disruption of mucus transport.

OVA-sensitised guinea pig tracheas were labelled with ^{35}SO to quantify mucosal output. OVA challenge significantly increases ^{35}SO output and this increase was similarly observed upon LTD_4 challenge. Both increases in mucosal outputs were inhibited by Pranlukast and Zafirlukast (177). In another study analysing mucus production in guinea pig trachea, challenged animals were euthanized and the dissected tracheas were stained with alcian blue-periodic acid Schiff to quantify mucus secretion (178). LTD_4 challenge increased mucus secretion which was inhibited by the cysteinyl leukotriene receptor antagonist, Pobilukast. In several studies using human airway mucosa explants it has been observed

that both LTC_4 and LTD_4 can increase mucus secretion which was inhibited by the cysteinyl leukotriene receptor antagonist, FPL55712 (179-181).

It has also been shown in the human airways that cysteinyl leukotrienes can affect mucus transport. It was observed in asthmatic individuals that the decrease in tracheal mucus velocity induced by antigen challenge could be inhibited by FLP55712 (182). LTC_4 and LTD_4 have been shown to significantly decrease human cilia beat frequency and similarly to the *in vivo* study, were inhibited by FLP55712 (183). However, in contrast to this study, scanning electron microscopy used to access cilia activity revealed that LTD_4 did not decrease cilia mobility but decreased orientation leading to cilia dysfunction (184).

The exact mechanism of how cysteinyl leukotrienes affect mucus production and transport has still to be elucidated but it has recently been shown in goblet cells isolated from rat and human conjunctiva that all cysteinyl leukotrienes can induce mucus secretion which was inhibited by MK-571 (185).

Bronchial hyperresponsiveness

Airway hyperresponsiveness (AHR) is the main characteristic feature of bronchial asthma and presents itself as an induction of bronchial constriction by inhalation of allergens or agonists that are not active on airways of healthy individuals. The role of cysteinyl leukotrienes in AHR is poorly understood. However, studies have shown that asthmatic airways are especially hyperresponsive to LTE_4 and this will be further discussed in section 1.1.7 *The third cysteinyl leukotriene receptor.*

1.1.6 Cysteinyl leukotrienes antagonists to treat asthma

A range of drugs exists that can be used in the treatment of asthma including inhaled corticosteroids, long-acting β_2 agonists and anti-leukotriene drugs. There are two classes of anti-leukotriene drugs; leukotriene synthesis pathway inhibitors and cysteinyl leukotriene receptor antagonists (LTRAs). Unfortunately, leukotriene synthesis inhibitors have been marred by adverse side effects: Zileuton and MK-0633 are inhibitors of the enzyme 5-lipoxygenase, and both have been shown to induce hepatotoxicity (186,187). In one trial of MK-0633, the study was permanently terminated due to dose-dependent increases in alanine aminotransferase and aspartate aminotransferase (187). GSK2190915 is a promising 5-lipoxygenase activating protein inhibitor that is already in phase II clinical trials and has shown no significant adverse effects on hepatic function

(188). LTRAs include Montelukast, Zafirlukast and Pranlukast which are selective $CysLT_1$ antagonists. Montelukast is generally the favoured LTRA in clinical use within the UK. There are no $CysLT_2$ antagonists in clinical use and the role of $CysLT_2$ in the pathogenesis of asthma is not clear.

Due to heterogeneity of asthma and patient to patient variability in response to treatment, certain guidelines have been introduced to aid management using first-line and add-on therapies (189).

First-line therapy

Inhaled corticosteroids are considered the standard first-line therapy in the treatment of asthma, irrespective of severity. LTRAs have been shown to significantly reduce exacerbations and improve symptom scores (compared to placebo) in chronic asthma but are generally considered inferior to corticosteroids and are mainly used as an add-on therapy (190,191). In a meta-analysis of randomised controlled trials where Montelukast was used as a therapy for asthma in adults, including 20 trials in chronic asthma and 6 trials in acute asthma, Montelukast was inferior at reducing exacerbations compared to inhaled corticosteroids alone, and to the combination of inhaled corticosteroids and long-acting β_2 agonists (192).

However, LTRAs are considered an acceptable first-line therapy for asthmatics under 5 who are unable to take inhaled corticosteroids (189). Several trials have been carried out comparing LTRAs with inhaled corticosteroids as first-line therapies for treatment of mild persistent asthma in children. Garcia *et al.* have compared Montelukast treatment with fluticasone (a corticosteroid) in a 52 week, multicentre, randomised, double blind trial in children aged 6-14 years old (193). Both therapies were well accepted and Montelukast increased to a similar extent the number of "rescue free days" (days without asthma rescue medication or asthma related resource use) as fluticasone (22.4 % vs. 25.2 %; Montelukast vs. fluticasone). Although, there were secondary end points that favoured fluticasone over Montelukast (for example, there were fewer incidences of asthma attacks and less requirement for systemic corticosteroids) it was noted that fluticasone treated children were on average significantly shorter in height after the 52 week trial compared to Montelukast.

In an open label, randomised, active-controlled, multicentre trial, Montelukast was compared to Budesonide (another inhaled corticosteroid) as a first-line therapy in children aged 2-4 years with mild persistent asthma (194). Similar to the Garcia *et al.* trial,

there were no significant differences in primary outcomes such as time to first additional medication, and both were considered an acceptable therapy for asthma control. However there were again higher incidences of the requirement for oral corticosteroids at 52 weeks in the Montelukast group compared to the Budesonide group.

Recently, Montelukast and Budesonide have been compared as a first-line therapy in a 12 week trial in children and adolescents aged 2-18 years (195). This was a multicentre, randomised and controlled trial with individuals exhibiting mild persistent asthma. There was a significant improvement in peak expiratory flow rates, FEV_1, symptom scores and exacerbation in both groups. However, in the Budesonide group there were significant increases in FEV_1 and daytime symptom scores compared to Montelukast.

Each of these trials is in agreement that Montelukast is not an inferior therapy to inhaled corticosteroids in the treatment of children with mild persistent asthma. Montelukast was considered an acceptable therapy for asthma control and although there were significant differences in FEV_1 and incidences in extra treatment (corticosteroids) compared to inhaled corticosteroids, this should be considered alongside the potential side effects that may occur through long-term use of steroids from a young age (such side-effects already displayed in Garcia *et al.*'s study, with significant differences in average height (193)).

Add-on therapy

LTRAs are commonly used as add-on therapies for uncontrolled asthma that cannot be maintained by inhaled corticosteroids. However, it has been noted in several studies that LTRAs can be used as an alternative to increasing inhaled corticosteroid dosage. In a randomised trial, single dose beclomethasone dipropionate (an inhaled corticosteroid) with Pranlukast was compared to double-dose beclomethasone dipropionate in adults with moderate asthma (196). Both therapies improved observed asthma control analysed by peak expiratory flow rate, symptom scores and addition of β_2 agonist treatment and there were no significant differences between the two treatments. These results have been observed in several trials analysing Montelukast plus budesonide versus double-dose budesonide (197,198). These studies revealed a new role of LTRAs in the treatment of asthma, especially in treatment of uncontrolled asthma and avoiding increase in inhaled corticosteroid dosage that have been linked to increases in local and systemic adverse effects (199).

LTRAs are prescribed generally for all types of asthma but there may be specific asthma phenotypes in which LTRAs could show more beneficial outcomes, for example, in

exercise-induced asthma. Almost half of asthmatics experience bronchial constriction induced by exercise. The cause of this is poorly understood but cysteinyl leukotrienes have been reported to increase in exhaled breath condensates and in urine (measured by urinary LTE_4) after exercise challenge (200,201). Several studies have indicated that Montelukast can significantly reduce exercise-induced bronchial constriction, as measured by FEV_1 (201,202).

Another phenotype that LTRAs could be particularly beneficial in is Aspirin-exacerbated respiratory disease (AERD) (also called aspirin-induced asthma (AIA) or aspirin-sensitive asthma (ASA)) which presents as severe bronchial constriction after exposure to aspirin and other non-steroidal anti-inflammatory drugs that inhibit cyclooxygenase-1. The prostaglandin and leukotriene synthesis pathway are thought to be in balance and inhibiting one side leads to the overproduction of the other. This appears to be the case in AERD, where inhibition of cyclooxygenase, the key enzyme in the synthesis of prostaglandins, shunts this balance to overproduce cysteinyl leukotrienes (203). Unfortunately, data are limited on long-term studies that analyse the effect of Montelukast treatment for AERD. Dahlen *et al*. ran a randomised double blind, placebo-controlled four week trial to analyse the addition of Montelukast treatment in AERD patients (204). They observed significant increases in pulmonary function and quality of life, and there was a 54 % decrease in incidences of exacerbations compared to placebo. This provides promising evidence that LTRAs could help manage AERD and other phenotypes that could be linked to cysteinyl leukotrienes. However, in general, long-acting β_2 agonists are considered the better add-on therapy although this could be different in some patient subsets.

1.1.7 The third cysteinyl leukotriene receptor

LTE_4 has become the "forgotten mediator" in leukotriene biology. This could be due to several reasons. Cloning of the $CysLT_1$ and $CysLT_2$ receptors revealed that LTE_4's affinity for these receptors was far weaker than those of LTC_4 and LTD_4 (see section 1.1.4 *Cysteinyl leukotriene receptors*). LTE_4's maximal response was more than 50 % lower than responses to LTC_4 and LTD_4 and thus it was labelled a partial agonist. Secondly, $CysLT_1$ cysteinyl leukotriene antagonists such as Montelukast were successfully introduced as treatments for asthma. Montelukast's ability to improve asthmatic symptoms, coupled with LTE_4's weak affinity has skewed research towards $CysLT_1$-mediated responses which are thought to be largely attributed to LTD_4. However, evidence from early studies has shown that LTE_4 is a potent agonist in its own right. Recent reports have speculated that

this could be due to LTE_4 preferential responses acting through a previously unidentified cysteinyl leukotriene receptor.

Early pharmacological data has shown that LTC_4, LTD_4 and LTE_4 (purified products of the slow-reacting substance) were able to elicit potent contractile responses on both guinea pig tracheal spirals and parenchymal strips with molar ratios of 1:1:0.1 and 1:0.05:0.3 respectively (LTC_4:LTD_4:LTE_4) (205). This reversal in potencies was one of the first indications that there were separate receptors for each leukotriene that were potentially expressed in the lungs. This was further supported by Lee *et al.*'s observations in tracheal smooth muscle of guinea pigs. LTE_4 was able to enhance airway responsiveness to histamine, but LTC_4 and LTD_4 were not, despite all three leukotrienes inducing constriction of the tracheal tissue to the same degree. Indomethacin, a non-selective inhibitor of cyclooxygenase, was able to attenuate LTE_4's augmentation of histamine reactivity but had no effect on LTE_4's contractile response, suggesting that LTE_4's hyperirritability was not completely associated with its contractile response (206). Removal of epithelium from guinea pig tracheal strips had no effect on LTE_4-mediated contraction. However, it did increase sensitivity to histamine 3-fold, but LTE_4 pretreatment did not modify histamine airway responsiveness (207,208). This suggested that the loss in LTE_4 augmentation of histamine responses could be either due to epithelium mediating tissue sensitivity or due to maximal tissue sensitivity being reached as a consequence of epithelium removal and so no further increase in sensitivity was possible.

Human studies have complemented the observations found in guinea pigs. *In vitro*, LTE_4 has been shown to be just as potent as LTD_4 at eliciting contraction in small bronchioles, and pretreatment of bronchial tissue with LTE_4 enhanced the tissues responsiveness to histamine (207,209). In the first *in vivo* study comparing inhaled LTE_4 responses to histamine-induced responses, LTE_4 was 39 times more potent than histamine in normal subjects (210). On comparison of cysteinyl leukotriene and histamine responses between asthmatic and healthy subjects, a disproportionate augmentation in relative responses to cysteinyl leukotriene inhalation in asthmatic subjects was observed (Table 1.6) (211).

Table 1.6 The relative fold changes in airway responsiveness between normal and asthmatic subjects upon inhalation of the indicated agonists

Agonist	Relative fold change between normal and asthmatic subjects
Histamine	13.6
LTC_4	6.2
LTD_4	9.1
LTE_4	219

This phenomenon is extended in AERD patients. Inhalation of LTE_4 was 1870 times more potent than histamine in aspirin-sensitive asthmatics at eliciting bronchial constriction and only 145 times more potent than histamine in aspirin-tolerant asthmatic subjects (212). After desensitisation with aspirin there was a substantial decrease in responsiveness to LTE_4 and no change in responsiveness to histamine in aspirin-sensitive subjects. Comparisons with aspirin-tolerant asthmatics showed no change in responsiveness to both LTE_4 and histamine after desensitisation with aspirin (212).

Regulation of cysteinyl leukotriene production has been shown to be especially important in AERD patients. Urinary concentrations of LTE_4 have been used clinically as a direct measure of cysteinyl leukotriene production. About 6-8 % of cysteinyl leukotriene produced is excreted as LTE_4 (213). Basal levels of urinary LTE_4 are higher in aspirin-sensitive asthmatics compared to aspirin-insensitive asthmatics. Interestingly, this level of urinary LTE_4 is significantly increased upon aspirin challenge, suggesting a possible pathway of cysteinyl leukotriene production by cyclooxygenase inhibitors such as aspirin (214,215). Urinary concentrations of LTE_4 are also significantly higher in aspirin-tolerant asthmatics that have frequent asthmatic attacks, compared to the stable condition (215,216). Atopic asthmatics also show higher levels of urinary LTE_4 after bronchial provocation with allergen but not with histamine, and patients with exercise-induced asthma, a phenotype of asthma, had significantly higher levels of urinary LTE_4 post-exercise challenge (201,214).

LTE_4 can also induce cell recruitment. Inhalation of LTE_4 in humans significantly increases infiltration of eosinophils, neutrophils and lymphocytes into the lamina propria of the airway mucosa (171,217). In comparative studies, there is a significant increase in infiltration of eosinophils into the sputum and lamina propria induced by LTE_4 inhalation compared to LTD_4 suggesting that LTE_4 is a far more potent chemo-attractive agent(172). Treatment with the selective $CysLT_1$ antagonist, Zafirlukast, can inhibit LTE_4-induced

eosinophilia and airway obstruction suggesting that LTE_4 actions are mediated by $CysLT_1$ or a $CysLT_1$-like receptor (171).

$CysLT_3$?

LTE_4's potent nature and unique characteristics that distinguish it from LTC_4 and LTD_4 has further strengthened the idea of the existence of a third cysteinyl leukotriene receptor. As previously discussed, cysteinyl leukotrienes can mediate vascular permeability (see section 1.1.5 *Increased vascular permeability*). In a mouse model of vascular oedema, analysing ear thickness after intradermal injection of ligand, LTE_4 has been shown to be the most potent cysteinyl leukotriene that can induce vascular leakage (218). Knockout of neither $CysLT_1$ nor $CysLT_2$ was able to affect LTE_4-induced responses. In double deficient $CysLT_{1/2}^{-/-}$ knockout mice, vascular permeability was increased. This finding has been repeated in a low-dose OVA-induced asthma mouse model (170). Pulmonary inflammation, denoted by bronchial vascular inflammation and goblet cell metaplasia was significantly increased in LTE_4 challenged mice. Knockout of both $CysLT_1$ and $CysLT_2$ revealed that LTE_4-induced inflammation remained intact. Both these studies have identified an LTE_4 mechanism that is independent of the known cysteinyl leukotriene receptors, $CysLT_1$ and $CysLT_2$. Identification of this LTE_4 preferential receptor would lead to the elucidation of specific mechanisms that could aid our understanding of the pathogenesis of asthma and other related inflammatory disorders, and potentially lead to the development of more effective therapies.

1.2 Project Aims

Cysteinyl leukotrienes have been implicated in many of the key features of asthma. They are involved in mediating vascular permeability, smooth muscle contraction and proliferation, bronchial hyperresponsiveness, increased mucus secretion, decreased mucus transport and migration of inflammatory cells such as eosinophils and Th2 cells. LTE_4, the most stable of the cysteinyl leukotrienes, has been shown to induce constriction in human bronchi to a similar degree as the other cysteinyl leukotrienes and is even more potent at eliciting airway eosinophilia than LTD_4. LTE_4 has also been shown to selectively augment airway responsiveness to histamine and human airways have been shown to be more sensitive to LTE_4 stimulation when comparing healthy and asthmatic individuals than to LTC_4 and LTD_4. However, characterisation of the cysteinyl leukotriene receptors, $CysLT_1$ and $CysLT_2$, have presented LTE_4 as a weak, "partial" agonist, as intracellular signalling responses are significantly lower than what is induced by LTC_4 and LTD_4. These observations do not match with the pharmacological profile of LTE_4, as a ligand that is just as potent and in some cases, even more potent, than LTD_4, which strongly suggests that responses to LTE_4 could be mediated via a previously unidentified cysteinyl leukotriene receptor(s).

The aim of this project is to aid our understanding of LTE_4, as a potent, "full" agonist by trying to identify a novel cysteinyl leukotriene receptor, preferentially signalling in response to LTE_4. This will be achieved through various approaches as follows:

1. Characterising potential cysteinyl leukotriene receptors that have already been identified in mouse models of inflammation. Human recombinant overexpression systems and primary cells will be used to analyse whether LTE_4 directly induces signal transduction. Various assays that measure secondary signalling cues such as intracellular calcium mobilisation and cAMP will be employed to verify this.
2. LTE_4 has already been shown to exert potent effects on human mast cells. Using two human mast cell lines, LAD2 and LUVA, intracellular calcium mobilisation and gene expression in response to cysteinyl leukotriene stimulation will be analysed. Gene screening by microarrays will then be used to analyse differential GPCR expression in order to identify a potential LTE_4 specific receptor. Target GPCRs will be analysed in recombinant systems and shRNA technology used to verify any promising target GPCRs that respond to cysteinyl leukotrienes.
3. Sequencing data of any target GPCR gene will be analysed to ascertain whether any polymorphisms within the gene may alter receptor structure or functions.

4. Finally, LTE_4 signal transduction pathways within human mast cell lines will be comprehensively analysed to ascertain what signalling requirements are necessary for LTE_4 to act as a "full", potent agonist. This will be achieved by using specific signalling pathway antagonists and analysing their effect on LTE_4-induced gene expression and intracellular calcium mobilisation.

Chapter 2

Materials and Methods

2.1 Materials

Cell culture media and supplements were purchased from Invitrogen (UK) unless otherwise stated. All cysteinyl leukotrienes were obtained from Cayman Chemical (Ann Arbor, MI). ADP, 2-MeS-ADP, isoproterenol, forskolin and PMA were purchased from Sigma-Aldrich (UK). Antibiotics ampicillin, pen step, kanamycin and puromycin were also purchased from Sigma-Aldrich. All primers stated were designed using on-line program (Roche) and synthesized by MWG (Germany). All other reagents were purchased from Sigma-Aldrich unless otherwise stated. Antibodies and plasmids were purchased from various companies as stated in the relevant methodology sections.

2.2 Cell culture

HEK293T cells were a gift from Dr Susan John (King's College London). These cells were cultured in high glucose (4500 mg/L) Dulbecco's modified Eagle's medium (DMEM) supplemented with glutamine (2 mmol/L), 10 % foetal bovine serum (FBS) and penicillin/streptomycin (50 units/ml / 50 ug/ml) in a 37^{o}C humidified 5 % CO_2 incubator. Cells were passaged every 3-4 days replacing all medium with fresh cell culture medium. Cells were counted using NucleoCounter (Chemometec).

LAD2 cells (a kind gift from Dr. Arnold Kirshenbaum, NIAID, NIH, USA; (19)) were cultured in StemPro-34 SFM supplemented with StemPro-34 nutrient supplement, penicillin/streptomycin (50 units/ml / 50 ug/ml), L-glutamine (200 mmol/L) and stem cell factor (SCF; 100 ng/ml) in a 37^{o}C humidified 5 % CO_2 incubator. Cells were hemidepleted weekly by replacing half the medium with equal volumes of fresh culture medium ensuring cell growth did not exceed 0.5x10^{6}/ml.

LUVA cells (a kind gift from Dr. John Steinke, University of Virginia, USA; (219)) were cultured in StemPro-34 SFM supplemented with StemPro-34 nutrient supplement, penicillin/streptomycin (50 units/ml / 50 ug/ml) and L-glutamine (200 mmol/L) in a 37^{o}C humidified 5 % CO_2 incubator. Cells were hemidepleted twice a week ensuring cell growth did not exceed 0.5x10^{6}/ml.

2.3 Plasmid preparation

3xHA tagged CYSLTR1 used in Figure 6.2B-D was cloned into specific plasmids suitable for lentiviral transfections. All other vectors shown in Table 2.1 were purchased from commercial sources.

Polymerase chain reaction (PCR). PCR was carried out to amplify 3xHA tagged CYSLTR1 that was within a pcDNA3.1+ plasmid (UMR cDNA Resource Centre) using Platinum Taq Polymerase High Fidelity (Invitrogen) following manufacturers protocols. Primers were custom designed containing restriction enzyme sites for NheI and BamHI. These were made by Eurofin MWG (5'-AGTGCTAGCATGTACCCATACGATGTT-3' and 5'-GCGGGGATCCCTATACTTTACATATTTC-3'). Reactions were performed under the following conditions; 94^{o}C for 1 minute, followed by 35 cycles of 94^{o}C for 30 seconds, 50^{o}C for 30 seconds and 68^{o}C for 30 seconds, finally 68^{o}C for 10 minutes using a Veriti 96 well thermal cycler (Applied Biosystems).

Purification of PCR product. PCR products were identified and purified on a 1 % agarose gel using ethidium bromide in a 1x tris-acetate-EDTA (TAE) buffer. Samples were run alongside either DNA ladder φX174 DNA/BsuRI (HaeIII) (φ, Thermo Scientific) or λ DNA/EcoRI+HindIII (λ, Thermo Scientific). Gels were imaged using ChemiDoc™ MP imager (Bio-RAD). Appropriate bands were cut and purified by QIAquick gel extraction kit (Qiagen) following manufacturers protocol and eluted in the supplied EB reagent.

Digestion. The purified PCR product and target plasmid, pCDH (encoding GFP, ampicillin and puramycin resistance under EF1 promoter and a multiple cloning site under CMV promoter (System Biosciences) were digested twice using NheI and BamHI (Thermo Scientific) following manufacturers protocols. Digestions were performed under the following conditions; overnight incubation at 37^{o}C then finally 65^{o}C or 80^{o}C (NheI or BamHI) for 20 minutes. After digestion the plasmid was dephosphorylated using FastAP phosphatase (Thermo Scientific) following manufactures protocols. Reactions were performed at 37^{o}C for 10 minutes then 65^{o}C for 15 minutes. All DNA was purified using QiaQuick gel extraction columns following the manufacturers PCR purification protocol and was eluted in the supplied EB reagent. DNA concentrations were measured using NanoDrop ND 1000 spectrophotometer (Thermo Scientific) and ND-1000 software version 3.2.0.

Ligation. The digested PCR product and plasmid were ligated using T4 Ligase (Thermo Scientific) following manufacturers protocols. In brief, the digested 3xHA tagged CYSLTR1 and pCDH plasmid were ligated at a 16:1 ratio (insert:plasmid) under the following

reaction conditions; 22°C for 20 minutes and 65°C for 10 minutes. Products were purified on a 1 % agarose gel following section 2.3 *Purification of PCR product* protocol.

Plasmid amplification. Plasmids containing genes of interest were amplified by transforming Top10 chemically competent cells (Life Technologies) by heat shock at 42°C. After a 1 hour recovery period in Soc-medium (Life Technologies), cells were incubated on LB-agar plates (1 % w/v NaCl (VWR), 1 % w/v tryptone (BD), 0.5 % yeast (BD), 1.5 % agarose type II) supplemented with the appropriate antibiotic overnight at 37°C. Plates intended for use with cells transformed with mouse-$P2Y_{12}$ were supplemented with Kanamycin (10 μg/ml); all others were supplemented with Ampicillin (50 μg/ml). Colonies were amplified in LB medium (1 % w/v NaCl, 1 % w/v tryptone, 0.5 % yeast) supplemented with the previously indicated concentrations of antibiotics at 37°C. Plasmids were extracted and purified using an Endofree Plasmid Maxi kit (Qiagen) and concentrations were quantified using NanoDrop ND 1000. All plasmids were sequenced by Eurofins MWG Operon and stored at -20°C.

Table 2.1 Plasmid information.

Gene	Clone ID	Company	Species	Antibiotic
GNA15	GNA1500000	UMR cDNA Resource Centre*	Human	Ampicillin
CYSLTR1	CLR0100000	UMR cDNA Resource Centre	Human	Ampicillin
CYSLTR2	CLR0200000	UMR cDNA Resource Centre	Human	Ampicillin
ADRB2 (3xHA)	AR0B20TN00	UMR cDNA Resource Centre	Human	Ampicillin
P2RY12 (3xHA)	P2Y120TN00	UMR cDNA Resource Centre	Human	Ampicillin
P2RY12	MC204030	OriGene**	Mouse	Kanamycin
MAS1L	SC305709	OriGene	Human	Ampicillin
MRGPRX2	SC120221	OriGene	Human	Ampicillin
GPR65	GPR6500000	UMR cDNA Resource Centre	Human	Ampicillin
OXGR1	OXGR100000	UMR cDNA Resource Centre	Human	Ampicillin

* Missouri S&T cDNA Resource Centre (**www.cdna.org**); ** OriGene Technologies, Rockville, MD

2.4 Transient transfection of HEK293T cells

HEK293T cells cultured to above 60 % confluence were transiently transfected with a mixture of Lipofectamine 2000 (Life Technologies) and 24 µg (total) plasmid containing the gene of interest in serum free conditions (Opti-MEM, Life Techologies) according to the manufacturer's protocol. After a 5 hour incubation at 37^{o}C, the transfection medium was removed and the cells were cultured for a further 36 hours in standard culture medium at 37^{o}C in a humidified 5 % CO_2 incubator.

2.5 Preparation of lentiviral particles and transduction of human mast cells

Glycerol stocks of shRNA cloned into pGIPZ vectors (Thermo Scientific) were amplified in standard LB medium (see section 2.3 *Plasmid preparation*) supplemented with ampicillin (50 µg/ml) and purified using Endofree Plasmid Maxi kit (Table 2.2). Insertion of the shRNA component into the amplified pGIPZ vectors were validated by a KpnI restriction digest run on a 1 % agarose gel.

Preparation of lentiviral particles. Plasmids containing either shRNA or 3xHA tagged $CysLT_1$ were transfected into HEK293T cells using $CaCl_2$, In brief, a mixture of the plasmid, with packaging vectors psPAX2, pDM2.G (System Biosciences) and $CaCl_2$ were bubbled through HEPES buffered saline (HBS). The transfection mixture was added to actively growing HEK293T cells and incubated for 24 hours at 37^{o}C in a humidified 5 % CO_2 incubator. Medium was replaced with fresh standard culture medium and HEK293T cell supernatants were collected at 24 and 48 hour time points. Supernatants were centrifuged at 200 g for 10 minutes and medium filtered (0.20 µm) to ensure no cellular contamination. Virus was precipitated by a 12 hour incubation at 4^{o}C with PEG-it solution (x1, System Biosciences) and collected by a centrifugation at 1500 g for 30 minutes. Viral pellets were resuspended in culture medium of target cells.

Transduction of LAD2 and LUVA cells with lentiviral particles. LAD2 cells (1x10^{6}) or LUVA cells (2x10^{5}) were hemidepleted and seeded in 6 well plates 24 hours prior to transfection. According to the manufacturer's protocol, virus was added directly to the LAD2 cells and incubated for 24 hours at 37^{o}C in a humidified 5 % CO_2 incubator. Medium was replaced with fresh culture medium and cells were incubated for a further 48 hours. Transfection efficiency was assessed by analysing GFP expression using flow cytometry (see section 2.14 *Flow cytometry*). Cells were then puromycin (2 µg/ml) selected to allow

only the cells that had incorporated the plasmid to continue growing. GFP expression of the cell line was monitored every 1-2 weeks using flow cytometry.

Table 2.2 pGIPZ shRNA specifications

Clone ID	Target	Accession	Mature Antisense
V3LHS_305475 (475)	CYSLTR1 Human	NM_006639	5'-TTAGTTTGATTGTCTTGTG-3'
V3LHS_305478 (478)	CYSLTR1 Human	NM_006639	5'-TACCTACACACACAAACCT-3'
V2LHS_90946 (946)	CYSLTR1 Human	NM_006639	5'-TCATGTATACTTGGAAGGC-3'
V2LHS_90947 (947)	CYSLTR1 Human	NM_006639	5'-TAGAGGTTGACATACAAAG-3'
V2LHS_111190 (190)	CYSLTR2 Human	NM_020377	5'-TTCAATTGTGCAGTTCCTG-3'
V3LHS_642322 (322)	CYSLTR2 Human	NM_020377	5'-ACATGTTGACATACAAGGA-3'
V3LHS_642324 (324)	CYSLTR2 Human	NM_020377	5'-AGAGCAGAGGATTGAAGCA-3'
V3LHS_642328 (328)	CYSLTR2 Human	NM_020377	5'-TCAGCTCTAAGCATGATGT-3'

2.6 Isolation of platelet-rich plasma

Blood was collected over citrate-dextrose solution (ACD, 6:1) from healthy volunteers who had given informed consent according to the regulations of the Research Ethics Committee of Guy's Hospital. Platelet-rich plasma (PRP) was prepared by centrifugation at 150 g for 15 minutes at room temperature. The upper layer was collected (PRP) and centrifuged for a further 5 minutes at 150 g to reduce erythrocyte contamination.

2.7 Intracellular calcium mobilisation assay

Calcium mobilisation assays were conducted using FLIPR calcium 4 assay kit and FlexStation 3 (Molecular Devices, Sunnydale, CA). The changes in fluorescence (Excitation – 485 nm, Emission – 525 nm, Emission Cut-Off – 515 nm) were recorded as relative fluorescence units (RFU) at 37°C every 4 seconds over selected time course with compound loading at 17 seconds.

Analysing transiently transfected HEK293T cells. HEK293T cells ($1.5x10^5$/well) were plated into poly-D-lysine (0.01 % w/v) coated 96 well plates in RPMI 1640 supplemented with HEPES (10 mmol/L). After a 5 hour incubation at 37°C, cells were further incubated for 1 hour with FLIPR loading buffer and fluorescent intensity was measured at 37°C using a

Flexstation 3 (Molecular Devices). Results were analysed with SoftMax Pro software version 2.4 (Molecular Devices).

Analysing isolated human platelets. PRP was washed in modified Tyrode's buffer (pH 6.2, NaCl 150 mol/L, KCl 3 mmol/L, glucose 5 mmol/L, $MgCl_2$ 1 mmol/L, HEPES 10 mmol/L, 0.1 % BSA) supplemented with PGE_1 (0.5 µmol/L). Platelets ($20x10^6$) were plated into 96 well plates in modified Tyrode's buffer supplemented with $CaCl_2$ (1.26 mmol/L) and incubated for 30 minutes with FLIPR loading buffer supplemented with probenecid (2.5 mmol/L). Florescence was measured using a Flexstation 3 machine at 37°C.

Analysing human mast cell lines. LAD2 or LUVA cells ($1.5x10^5$/well) were plated on poly-L-lysine (0.01 % w/v) coated 96 well plates in RPMI supplemented with HEPES (10 mmol/L) and incubated with FLIPR loading buffer for 1 hour at 37°C. Fluorescent intensity induced upon stimulation was measured at 37°C using a Flexstation 3 (Molecular Devices).

2.8 cAMP accumulation assay

Analysing transiently transfected HEK293T. Intracellular cAMP accumulation was analysed in HEK293T cells using a Cyclic AMP assay kit (Meso Scale Discovery, Gaithersburg, MD, USA) following the manufacturer's protocol. HEK293T cells with the addition of IBMX (1 µmol/L) were plated on anti-cAMP coated MULTI-ARRAY 96 well small spot plates (MSD), stimulated with forskolin and agonists for 15 minutes as indicated, lysed and run on the ImageSector 6000 (MSD). Results were analysed using MSD workbench software (MSD).

Analysing isolated human platelets. Intracellular cAMP accumulation was analysed in PRP using the HitHunter cAMP XS+ assay kit (DiscoverX, UK) following the manufacturer's protocol. PRP was centrifuged at 1000 g for 10 minutes and washed three times with pre-chilled DPBS supplemented with EDTA (2 mmol/L). Platelets ($2.5x10^8$) suspended in DPBS supplemented with EDTA (2 mmol/L) and IBMX (1 µmol/L), were plated on a 96 well plate, stimulated with forskolin and other agonists for 15 minutes as indicated and incubated with detection reagents. Luminescent signal was measured 4 hours after lysis using a Flexstation 3. Results were analysed with SoftMax Pro Software.

2.9 β-arrestin recruitment assay

Analysis of β-arrestin recruitment was conducted using a PathHunter eXpress β-arrestin kit (DiscoverX) following the manufacturer's protocol. In this system, the GPCR and β-arrestin are fused to two fragments of β-galactosidase and the interaction of the two proteins results in an enzymatic reaction. In brief, CHO cells stably transfected with the C-terminally modified human or mouse $P2Y_{12}$ receptor and with the β-arrestin, N-terminally tagged with deletion mutant of β-galactosidase, were seeded on 96 well plates in OCC medium for a 48 hour recovery period at 37°C. Cells were stimulated for 90 minutes at 37°C as indicated and then incubated with detection reagents for a further 90 minutes at room temperature. Luminescent signal, which is directly related to the recruitment of β-arrestin to $P2Y_{12}$ in the assay, was measured using Flexstation 3 and SoftMax Pro Software.

2.10 Analysis of gene expression

Stimulation of human mast cell lines. LAD2 or LUVA cells ($5x10^5$) in SCF free conditions were treated for 30 minutes with L-cysteine (3 mmol/L). In some experiments cells were preincubated for indicated time points prior to ligand addition with inhibitors. Cells were stimulated for 2 hours with indicated concentrations of ligand and then were centrifuged at 200 g for 5 minutes. RNA was isolated from the pellets and mRNA expression levels were analysed by qRT-PCR, see section 2.11 *RNA isolation and analysis*.

Analysis of gene expression profiles of LUVA cells by microarray. LUVA cells ($5x10^5$) were treated for 30 minutes with L-cysteine (3 mmol/L) at 37°C, then stimulated for 2 hours with a vehicle control (medium/EtOH) at 37°C. Cells were then centrifuged for 5 minutes at 200 g and RNA was isolated, see section 2.11 *RNA isolation and analysis*. RNA was analysed using Affymetrix whole transcriptome expression arrays (Human Gene 1.0 ST Arrays; Affymetrix). Sense-strand cDNA was generated using the Ambion Whole Transcriptome (WT) expression kit (Applied Biosystems) following the manufacturer's protocol. RNA and cRNA yield and size distribution were analysed on an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) using nanofluidic chips (Agilent Technologies). Results were analysed on Agilent 2100 software revision B.02.02 (Agilent Technologies). cRNA was fragmented and labelled using Affymetrix GeneChip WT terminal labelling and hybridization kit (Affymetrix) following the manufacturer's protocol. Chips were run by FWB Genomics Facilities (King's College London) using an Affymetrix

GeneChip Scanner 3000 (Affymetrix). Results were analysed using the Partek Genomics Suite software.

2.11 RNA isolation and analysis

RNA extraction and quantification. Cells lysed in Trizol (Life Technologies) were homogenised further using QIAshredder columns (Qiagen). Total RNA was extracted and purified using an RNeasy mini kit (Qiagen) and an intermediate TURBO DNase (Ambion) treatment following the manufacturer's protocol to ensure complete genomic DNA removal. RNA concentrations were measured using NanoDrop ND 1000 spectrophotometer and ND-1000 software version 3.2.0. All samples were stored at -80^{o}C.

Random hexamer primed reverse transcription. Reverse transcription was carried out using RevertAid M-MuLV reverse transcriptase (Fermentas) and primed using random hexamers according to the manufacturer's protocol. In brief, RNA was first incubated with random heaxmers in a Veriti 96 well thermal cycler for 5 minutes at 65^{o}C. Primed RNA was then incubated with a mixture of 1x M-MuLV reaction buffer, dNTPs, Ribolock RNase inhibitor and RevertAid H Minus reverse transcriptase (all Fermentas) for sequential incubation steps of 10 minutes at 25^{o}C, 60 minutes at 42^{o}C and 10 minutes at 70^{o}C. All cDNA samples were stored at -20^{o}C.

Quantitative real time polymerase chain reaction (qRT-PCR) qRT-PCR was carried out on an ABI PRISM 7900HT fast real-time PCR system (Applied Biosystems) and relative quantification studies were performed using SDS 2.4 and RQ Manager 1.2.1 software (Applied Biosystems) for Chapter 3-4 all other qRT-PCR experiments were carried out on a ViiA 7 PCR (Applied Biosystems) and analysed using ViiA 7 software version 1.0 (Applied Biosystems). mRNA expression levels were analysed in triplicate using relative quantification with 18s rRNA as the endogenous control. All UPL probes (Roche) and primers (synthesised by MWG Operon) were FAM labelled and designed at www.roche-applied-science.com and previously tested for optimal efficiency of PCR amplification. See Table 2.3 for probe and primer specifications.

Table 2.3 qRT-PCR probe and primer specifications

Gene	Probe	Forward Primer	Reverse Primer
CYSLTR1 (Human)	5'-CTGGCTGC-3' (#71)	5'-GGAGAGGGTCAAAGCAACAA-3'	5'-TGCAGAAGTCCGTGGTCATA-3'
CYSLTR2 (Human)	5'-TGGCTCTG-3' (#21)	5'-TGATGTGACACTGCCGTTCT-3'	5'-TCATGGCTTCCTCAATAATGC-3'
P2RY12 (Human)	5'-CAGGCAGC-3' (#27)	5'-GGAAAGAGCATTTCTTCACATTCT-3'	5'-TTTGCCTAACATGATTCTGACC-3'
P2RY12 (Mouse)	5'-TGCTGGGC-3' (#107)	5'-AAATCCTCATTGCCAAGCTG-3'	5'-TGCTGTACACCGTCCTGTTC-3'
CCL4 (Human)	5'-CCAGCCAG-3' (#20)	5'-CAGCACAGACTTGCTTGCTT-3'	5'-CTTCCTCGCAACTTTGTGGT-3'
PTGS2 (COX2) (Human)	5'-CTTCCTCC-3' (#69)	5'-AGTCGGGCAATCATCAGG-3'	5'-TGGGAAGCCTTCTCTAACCTC-3'
CSF2 (GMCSF) (Human)	5'-CCTGGAGC-3' (#1)	5'-GCCCTTGAGCTTGGTGAG-3'	5'-TCTCAGAAATGTTTGACCTCCA-3'

A comparative Ct method was used to analyse the data obtained. All samples were first normalised to an appropriate endogenous housekeeping gene (18s). ΔCt of samples of interest were then compared to a calibrator sample to acquire the ΔΔCt.

$Ct_{\text{target gene}} - Ct_{\text{endogenous gene}} = \Delta Ct$

$\Delta Ct_{\text{sample}} - \Delta Ct_{\text{calibrator}} = \Delta\Delta Ct$

Relative mRNA expression levels of the samples of interest were then expressed as $2^{-\Delta\Delta Ct}$.

2.12 Analysis of protein expression by ELISA

Stimulation of isolated human platelets. PRP supplemented with PGE_1 (0.5 μmol/L) was centrifuged at 1000 g for 10 minutes and washed three times with pre-warmed modified Tyrode's buffer supplemented with PGE_1 (0.5 μmol/L). PRP was stimulated for 15 minutes with indicated concentrations of ligand, centrifuged at 1000 g for 10 minutes and supernatant was collected and stored at -20°C.

Analysis of supernatants by ELISA. CCL5 was measured in supernatants using human CCL5/RANTES duo set kits (R&D Systems, UK) following the manufacturer's protocol. In brief, plates coated overnight with the capture antibody were washed three times with wash buffer (0.05 % Tween in PBS) and incubated overnight at 4°C with the supernatants of interest. Plates washed three times with wash buffer were incubated for 2 hours with the detection antibody. After a further three washes plates were incubated with streptavidin-HRP for 20 minutes and then washed for a final three times. Plates were then incubated for 15 minutes with TMB single solution (Life Technologies), a substrate for HRP and reactions stopped with HCl (1 N). Optical density was recorded on Anthos htIII (Anthos Labtech) using Stringray software (DazDaq). Measurements at 450nm were corrected by measurements at 578nm and protein concentrations were generated from the standard curve.

2.13 Analysis of protein expression by Western blot

Stimulation of human mast cell lines for Erk activation. LAD2 or LUVA cells ($5x10^5$) in SCF free conditions were stimulated for indicated times with 100 nmol/L of ligand and stimulations were stopped by the addition of chilled PBS. Cells were centrifuged at 200 g for 5 minutes at 4°C and pellets were processed as stated below.

Protein extraction. Pellets were lysed with a Western lysis buffer (50 mmol/L Tris.HCl, 1 mmol/L EDTA, 1 % TritonX-100) supplemented with protease and phosphotase inhibitors (Roche) and homogenised by vortexing. Lysates were incubated on ice for 30 minutes and homogenised further on a rotary mixer for a further 30 minutes at 4^{o}C. After a centrifugation at max speed for 20 minutes, supernatants were stored at -20^{o}C. Protein concentrations were quantified using a BCA protein assay kit (Thermo Scientific) and run on Anthos htIII using Stingray software. Absorbance was measured at 560 nm and protein concentrations were generated from the standard curve.

Western Blotting Proteins (20 µg) were denatured at 90^{o}C for 10 minutes in the presence of NuPAGE sample reducing agent and NuPAGE LDS sample buffer. Samples and an appropriate ladder (Precision Plus, Bio-Rad, Richmond, CA) were loaded onto a NuPAGE Novex 10 % Bis-Tris 1.0 mm gel and run at 180 V for 1 hour in NuPAGE MES SDS running buffer supplemented with antioxidants. Proteins were transferred onto a nitrocellulose membrane (0.2 µm pore size) at 30 V for 1 hour in NuPAGE transfer buffer supplemented with antioxidants and then washed three times with TBS 0.05 % Tween (TTBS; Sigma-Aldrich). Membranes were blocked for 2 hours with 5 % milk in TTBS and then incubated with the primary antibody overnight at 4^{o}C (Table 2.4). After membranes were washed with TTBS, they were incubated with the secondary antibody for a further 1.5 hours at room temperature (Table 2.4). After a final wash in TTBS, membranes were developed using ECL plus detection reagents (GE Healthcare) and processed on a ChemiDoc MP (Bio-Rad) using Image Lab software version 4.1 (Bio-Rad).

Membranes were stripped using Restore PLUS Western blot stripping buffer (Thermo Scientific) in order to probe for the control protein. In brief, membranes washed with TTBS were incubated with the stripping buffer for 10 minutes at room temperature. Membranes were washed again with TTBS and blocked for 2 hours with 5 % milk in TTBS. Re-probing for the control protein was carried out according to the protocol above.

Table 2.4 Primary and secondary antibodies used for Western blotting.

Antibody	Clone	Host	Company
COX-2 Monoclonal	CX229	Mouse	Cayman Chemical
GAPDH Monoclonal	6C5	Mouse	GeneTex*
Phospho-p44/42 MAPK	T202/Y204	Rabbit	Cell Signaling Technologies**
p44/42 MAPK Polyclonal	-	Rabbit	Cell Signaling Technologies
Goat anti-mouse IgG-HRP	-	Goat	Southern Biotech***
Goat anti-rabbit IgG HRP	-	Goat	Southern Biotech

* GeneTex Inc., San Antonio, TX, USA; ** Cell Signaling Technologies, Beverly, MA, USA; *** Southern Biotech, Cambridge, UK.

2.14 Flow cytometry

Analysis of GPCR surface expression. Transiently transfected HEK293T cells were stained with an Alexa Fluor 488 conjugated anti-HA monoclonal antibody (Clone 16B12, Covance, Ca) that recognises the HA epitope that is N-terminally located on the receptors of interest. In brief, cells washed twice with PBS were stained and incubated in the dark at room temperature for 20 minutes. After a further two washes with PBS, cells were analysed using the FL1 (530/30) laser on a FACScalibur with CellQuest Pro software (BD Biosciences).

Analysis of platelet activation. Whole peripheral blood drawn over ACD (6:1) was stimulated with the indicated ligands immediately after collection for 10 minutes at room temperature. 5 µl of blood was directly stained with monoclonal antibodies against CD61 (APC, clone VI-PL2) and CD62P (PE, clone Psel.KO2.3) or appropriate isotype controls (all eBiosciences) and incubated in the dark at room temperature for 20 minutes. Cells were fixed with 1 % formaldehyde and analysed using the FL2 (585/42, PE) and FL4 (661/16, APC) lasers on the FACScalibur. Signals were compensated accordingly.

Analysis of lentiviral transduced LAD2 cells. Transfection efficiency of transduced LAD2 cells was assessed by analysing GFP expression. In brief, transduced and untransduced LAD2 cells washed twice with PBS were stained with a fixable viability dye; eFluor 780 (eBiosciences) and incubated in the dark on ice for 20 minutes. After a further two washes

with PBS, cells were fixed in 2 % formaldehyde and analysed using the FL1 (530/30, GFP) and FL3 (670, eFluor 780) lasers on a FACScalibur. No compensation was necessary.

2.15 DNA isolation and analysis

DNA extraction and quantification. Total DNA was extracted and purified by DNeasy Tissue kit (Qiagen) following manufacturers protocol for cultured cells. DNA concentrations were measured using NanoDrop ND 1000 spectrophotometer and ND-1000 software version 3.2.0. All samples were stored at -80°C.

PCR and purification. PCR was carried out to amplify specific fragments of the CYSLTR1 gene using Platinum Taq Polymerase High Fidelity (Invitrogen) following manufacturers protocols. Primers were custom designed and made by Eurofin MWG (see Table 2.5 for primer information). Reactions were performed under the following conditions; 94°C for 1 minute, followed by 35 cycles of 94°C for 30 seconds, 63°C or 58°C for 30 seconds (CYSLTR1 promoter or coding region amplification, respectively) and 68°C for 30 seconds, finally 68°C for 10 minutes using a Veriti 96 well thermal cycler. Purification of DNA was performed on a 1 % agarose gel, see section 2.3 *Purification of PCR product*.

Table 2.5 Primer specifications for CYSLTR1 promoter and CDS amplification.

Target	Orientation	Sequence
CYSLTR1 promoter	Forward	5'-AACTGGAGACTTGCAGGTTGCG-3'
CYSLTR1 promoter	Reverse	5'-AACATCAAAGTGCTGCCCCAGG-3'
CYSLTR1 CDS	Forward	5'-TCAATGCCTCACTACTATTGCTTG-3'
CYSLTR1 CDS	Reverse	5'-TTGGTTTGGACTGGAAATGGG-3'

Gene coding region (CDS).

Sequencing. All DNA sample concentrations were adjusted to 1 ng/µl per 100 base pairs and sequenced using custom designed primers (see Table 2.6 for primer information) made by Source Biosciences. Sequencing was carried out by Source Bioscience Sanger sequencing service and analysed using BLAST analysis (NCBI).

Table 2.6 Primer specifications for CYSLTR1 promoter and CDS sequencing.

Target	Orientation	Sequence
CYSLTR1 promoter	Forward	5'-TAAGATGGGAAGCAGGGACG-3'
CYSLTR1 promoter	Reverse	5'-GGCTTCAATCAGCACATACC-3'
CYSLTR1 CDS	Forward	5'ATACCAAGTGCTTTGAGCC-3'
CYSLTR1 CDS	Forward	5'-GCATTTGGCTCTTTGGTG-3'
CYSLTR1 CDS	Reverse	5-'GTTTGATTGTCTTGTGGGG-3'

Gene coding region (CDS).

2.16 Statistical analysis

Appropriate data were analysed by means of one- or two- way ANOVA using GraphPad Prism software (GraphPad, La Jolla, CA). Differences were considered significant at a p-value less than 0.05.

Chapter 3

Analysis of potential novel cysteinyl leukotriene receptors

3.1 Introduction

$P2Y_{12}$ (or SP1999 as it was originally known) was identified as the elusive platelet ADP receptor that was sensitive to antithrombotic drugs, ticlopidine and clopidogrel (220-222). Using two completely separate approaches to GPCR identification, one using the ligand to identify the receptor and the reverse, using the receptor to identify the ligand, the $P2Y_{12}$ $G\alpha_i$ signalling pathway that worked in concert with $P2Y_1$'s ADP activated $G\alpha_q$ signalling pathway was elucidated. Knocking out of either receptor, $P2Y_1$ or $P2Y_{12}$, in platelets within mouse models significantly affected platelet functionality by attenuating shape change and aggregation (221,223). Although deemed a complex interplay it is still debatable whether their mode of action is by heterodimerisation of the two receptors or just cross reciprocal talk (224).

$P2Y_{12}$ is part of the "purinergic" family of GPCRs that are activated by purines and pyrimidines and is mainly expressed on platelets and in the brain e.g. astrocytes (225). The purinergic family of GPCRs was first defined in 1976 by Geoffery Burnstock and later subdivided into P1 (adenosine) and P2 (ATP and ADP) receptor terminology (226,227). P2 receptors are further divided based on pharmacology into P2X (ionotropic receptors) and P2Y (metatropic receptors) (228). To date, there are eight P2Y subtype receptors that have been recognised as true P2Y receptors (229). The P2Y family contains: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$. Original nomenclature was defined on chronological order of cDNA cloning but even though some receptors had similar sequence homology, when characterised they did not respond to nucleotides and so were excluded from the P2Y family (229). For example, $P2Y_7$ was eventually characterised as a leukotriene B_4 receptor (LTB4R)and $P2Y_{15}$ was shown to be activated by the citric acid cycle intermediate, α-ketoglutarate (OXGR1) (230-232). $P2Y_5$ and $P2Y_{10}$ are still deemed to be orphan receptors and so are not currently included in this list.

In silico screening using the $P2Y_1$ and bovine rhodopsin as confirmation templates has revealed that $P2Y_{12}$ could also respond to LTE_4 (233). Nonaka *et al.*'s study showed that LTE_4 as well as other potential ligands within the nucleotide and lipid families have sufficient binding energies to potentially elicit activation of this purinergic receptor. Interestingly, in their league table of ligands with the highest binding energy, LTE_4 came 12 and 31 places higher than $P2Y_{12}$'s known ligands, 2-MeS-ADP and ADP respectively, showing that $P2Y_{12}$ may have the potential for dual ligand functionality, as $CysLT_1$ was initially described as activated by both nucleotide and lipid (125,140,233). When overexpressed in CHO cells with the fusion protein, $G\alpha_{16}$, Nonaka *et al.* were able to

observe intracellular calcium mobilisation in response to LTE_4 stimulation (but to no other cysteinyl leukotrienes) showing that LTE_4 could have the potential to activate $P2Y_{12}$ (233).

Paruchuri *et al.* analysed *in vivo* whether $P2Y_{12}$ was involved in LTE_4 mediated pulmonary inflammation (170). As previously described, their study showed that low dose OVA sensitised mice challenged intranasally with LTE_4 showed significant increases in BAL eosinophilia, bronchial vascular infiltration and goblet cell metaplasia when compared to the control mice. This potent induction of pulmonary inflammatory responses remained intact in LTE_4 challenged $CysLT_1/CysLT_2^{-/-}$ double knockout mice. $P2Y_{12}$ knockout as well as platelet depleted mice saw a substantial reduction in all LTE_4 mediated functions although direct binding of radiolabelled LTE_4 to $P2Y_{12}$ could not be demonstrated (170). Their study highlighted the involvement of $P2Y_{12}$ in the LTE_4-induced inflammatory cascade but it left unanswered a question whether LTE_4 is a direct $P2Y_{12}$ agonist.

OXGR1, previously known as GPR99 or $P2Y_{15}$, is a GPCR that was identified a decade ago as the receptor for the citric acid cycle intermediate, α-ketoglutarate (232). It is a receptor that shares sequence homology with the purinergic family of GPCRs and has been shown to signal by coupling to $G\alpha_q$ (232). OXGR1 has also been shown recently to recruit β-arrestin upon stimulation with α-ketoglutarate (234). General expression of OXGR1 within mouse tissue has been shown to be primarily located in the kidney, smooth muscle and the testis (232). Using OXGR1 knockout mice models, α-ketoglutarate acting through OXGR1 has been shown to be extensively involved in paracrine regulatory signalling of the acid-base balance within the cortical collecting duct and the connecting tubule of the kidney (235).

Recently OXGR1 has been suggested as a potential third cysteinyl leukotriene receptor that is preferentially activated by LTE_4 (236). Kanaoka *et al.* identified OXGR1 as a potential target by using a reporter gene expression assay containing P2Y related receptors that responded to dicarboxylic acids. As previously described, $CysLT_1/CysLT_2^{-/-}$ knockout actually increases LTE_4-induced ear oedema when compared to the wild type controls (237). Triple knockout mice, $OXGR1/CysLT_1/CysLT_2^{-/-}$, revealed a substantial attenuation of LTE_4, as well as LTC_4 and LTD_4,-induced ear oedema (236). This study strongly suggests the involvement of OXGR1 in cysteinyl leukotriene mediated vascular oedema.

3.2 Study Aims

Over the past decade several GPCRs have been highly speculated as the elusive third cysteinyl leukotriene receptor. $P2Y_{12}$ and OXGR1 are the most recent of these to be identified as potential LTE_4 receptors. Both receptors have been the subject of knockout experiments in mouse models of inflammation and showed that LTE_4-induced inflammation was severely abrogated in the absence of these receptors (170,236). However, no convincing evidence for direct binding or activation of either of those receptors by cysteinyl leukotrienes was provided in the studies.

The aim of this study is to elucidate whether LTE_4 is a direct agonist of either $P2Y_{12}$ or OXGR1. This will be achieved by comprehensive analysis of signalling events in heterologous overexpression models (HEK293T cells) and human platelets, a primary cell that constitutively expresses $P2Y_{12}$.

3.3 Results

3.3.1 Validation of the heterologous expression model in HEK293T cells

To ascertain whether LTE_4 could mediate signal transduction through the $P2Y_{12}$ receptor, a model of heterologous receptor expression was established in HEK293T cells. These cells do not natively express any known classical cysteinyl leukotriene receptors or $P2Y_{12}$ and when unmodified they do not respond to cysteinyl leukotriene stimulation (Figure 3.1). To validate this model, forward constructs of human (h)-$CysLT_1$ and h-$CysLT_2$ in pcDNA3.1 expression vectors where transiently transfected into separate HEK293T populations and verified by qRT-PCR (Figure 3.2A-B). Transfectants were stimulated with exogenous LTC_4, LTD_4 and LTE_4 and their intracellular responses were measured using FLIPR Calcium 4 assay kit and a Flexstation 3. Both [$CysLT_1$] and [$CysLT_2$] transfectants responded robustly with calcium mobilisation to cysteinyl leukotriene stimulation, with receptor potencies mimicking what has been previously published in literature (LTD_4>LTC_4>>LTE_4 and LTD_4=LTC_4>>LTE_4, respectively) (Figure 3.2C-D)(114,238,239). Pre-incubation with the selective $CysLT_1$ antagonists, MK-571 and Montelukast, completely abrogated cysteinyl leukotriene mediated calcium mobilisation in [$CysLT_1$] transfectants while having no effect on signalling in [$CysLT_2$] transfectants. Similarly, the selective $CysLT_2$ antagonist, HAMI 3379, diminished calcium mobilisation in [$CysLT_2$] transfectants but had no effect on [$CysLT_1$] signalling (Figure 3.2E-F).

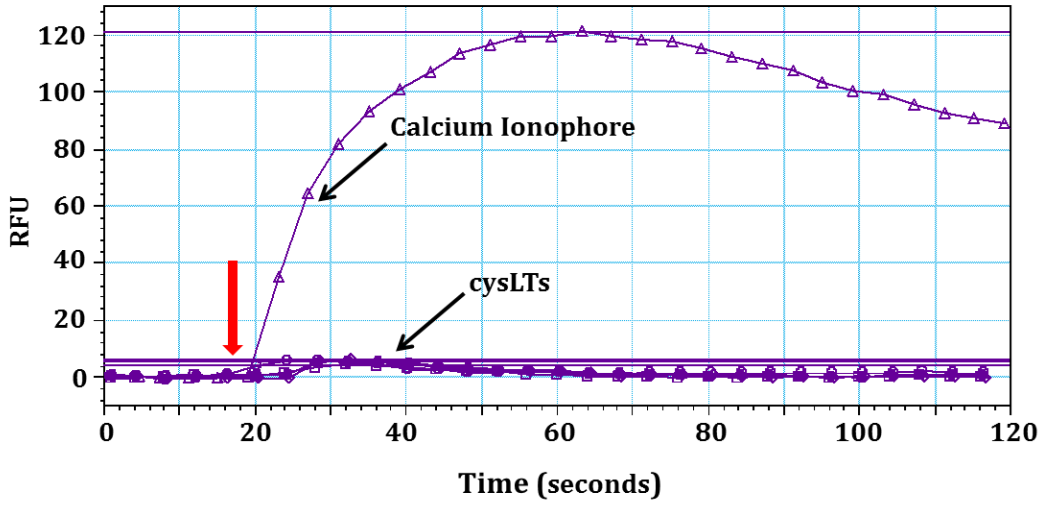


Figure 3.1 The effect of cysteinyl leukotriene stimulation on HEK293T.

Raw intracellular calcium flux data of empty vector transfected cells stimulated with 300 nmol/L exogenous LTC_4 (diamond), LTD_4 (square), LTE_4 (closed circle), vehicle control (open circle) and calcium ionophore, A23187, 1 µmol/L (triangle), representative of 3 experiments run in triplicate, red arrow indicates start of stimulation. Relative fluorescence units (RFU).

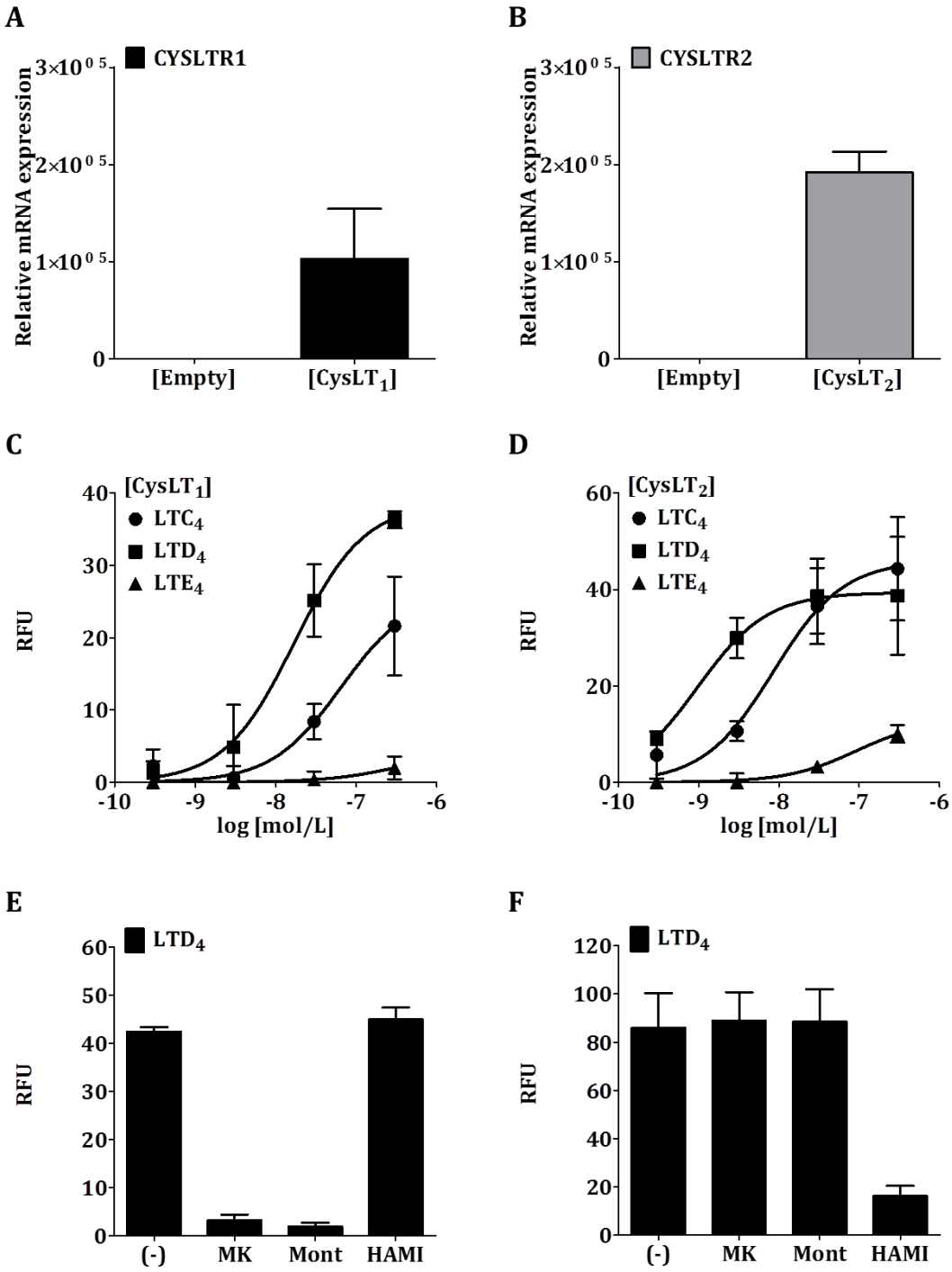


Figure 3.2 The effect of cysteinyl leukotriene stimulation on HEK293T cells transiently transfected with h-CysLT$_1$ and h-CysLT$_2$.

Forward constructs of h-CysLT$_1$ and h-CysLT$_2$ were transiently transfected into separate HEK293T populations by lipofection. Relative quantification of human **(A)** CYSLTR1 and **(B)** CYSLTR2 mRNA in [CysLT$_1$] and [CysLT$_2$] transfectants, respectively, compared to empty vector transfected HEK293T cells, 18s used as an endogenous control, *n*=3. Baseline corrected peak calcium fluxes in response to stimulation of **(C)** [CysLT$_1$] transfectants and **(D)** [CysLT$_2$] transfectants with indicated concentrations of exogenous LTC$_4$ (circle), LTD$_4$

(square) and LTE_4 (triangle), n=2 run in triplicate. Baseline corrected peak calcium fluxes in response to 100 nmol/L exogenous LTD_4 with or without a 15 minute pre-incubation with "MK" (MK-571) 1 μmol/L, "Mont" (Montelukast)100 nmol/L or "HAMI" (HAMI 3379)1 μmol/L of **(E)** [$CysLT_1$] transfectants and **(F)** [$CysLT_2$] transfectants, n=3 run in triplicate. Data shown as mean ± SEM. Relative fluorescence units (RFU).

3.3.2 Validation of functional overexpression of human $P2Y_{12}$ in HEK293T cells

As previously stated, HEK293T cells do not respond to cysteinyl leukotriene stimulation but it is important to ascertain whether they respond to ADP, the natural ligand of $P2Y_{12}$. 3xHA N-terminally tagged h-$P2Y_{12}$ and empty constructs in pcDNA3.1 vectors were transiently transfected into separate HEK293T populations. [h-$P2Y_{12}$] transfectants stained with an anti-HA antibody (for surface receptor expression) were considered 85.45 % ± 7.49 % positive for h-$P2Y_{12}$ expression when compared to the [Empty] controls (Mean ± SD, *n*=3, Figure 3.3A). There was also a substantial increase in P2RY12 mRNA in [h-$P2Y_{12}$] transfectants compared to [Empty] transfectants (Figure 3.3B), therefore validating the transfection procedure.

Stimulation of [h-$P2Y_{12}$] transfectants with exogenous ADP-induced potent intracellular calcium mobilisation that was similar to [Empty] transfectants (Figure 3.4A-B). Comparison of peak calcium flux data over a range of concentrations (10-0.01 μmol/L) revealed no statistically significant differences in both dose-dependent response curves to ADP (n.s., two-way ANOVA). These observations reflect constitutive expression of other purinergic receptors and that h-$P2Y_{12}$ signal transduction does not occur through calcium mobilisation in this model.

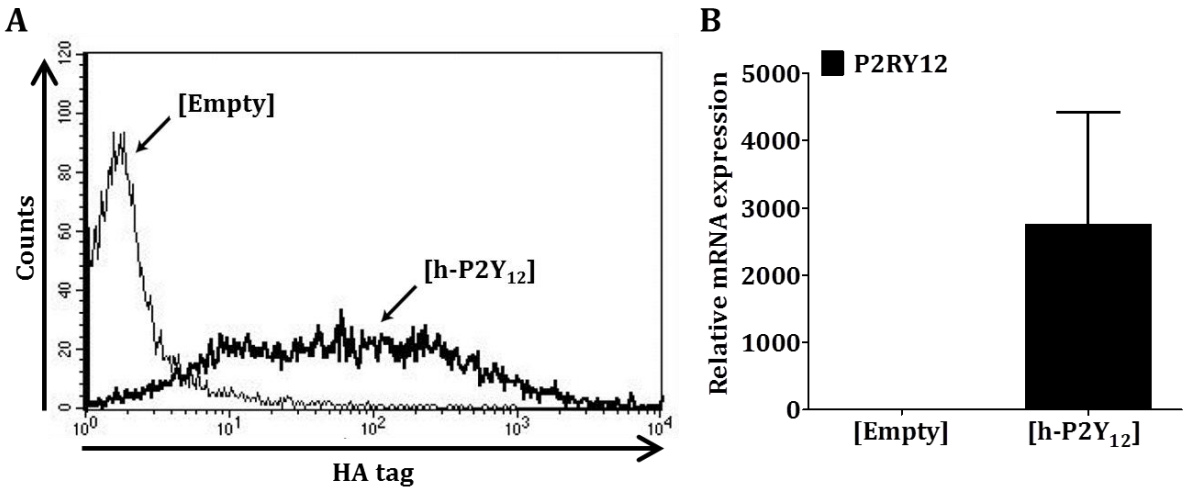


Figure 3.3 Validation of the h-P2Y$_{12}$ overexpression model.

Forward constructs of 3xHA N-terminally tagged h-P2Y$_{12}$ and empty constructs in pcDNA3.1+ expression vectors were transiently transfected into separate HEK293T populations by lipofection. **(A)** Flow cytometry analysis of h-P2Y$_{12}$ expression in [h-P2Y$_{12}$] and [Empty] transfectants using a Alexa Fluor labelled antibody specific for 3xHA, 1:1000, results from a single experiment representative of 3. **(B)** Relative quantification of human P2RY12 mRNA in [h-P2Y$_{12}$] compared to [Empty] transfectants, 18s used as an endogenous control, *n*=3. Data represented as mean ± SEM.

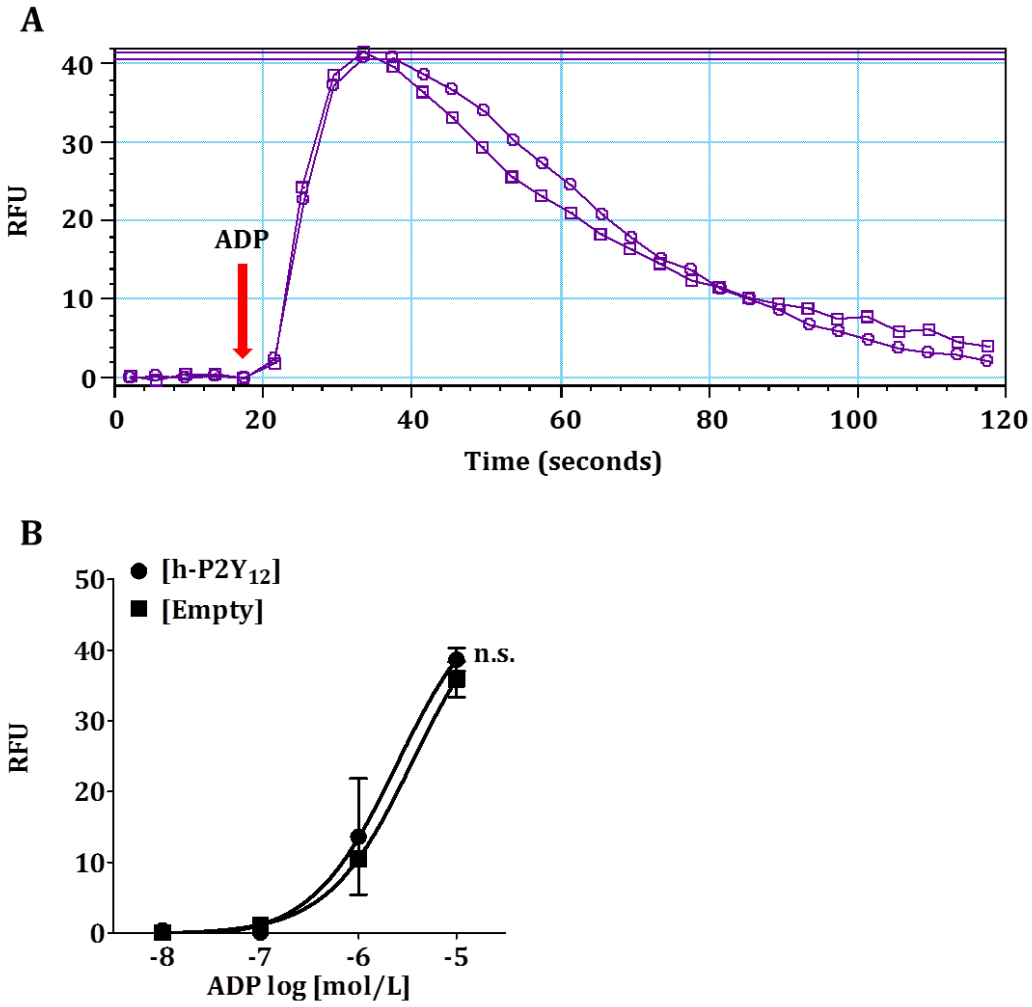


Figure 3.4 The effect of ADP stimulation on calcium mobilisation of h-P2Y$_{12}$ and empty vector transfected HEK293T cells.

Forward constructs of 3xHA N-terminally tagged h-P2Y$_{12}$ and empty constructs in pcDNA3.1+ expression vectors were transiently transfected into separate HEK293T populations by lipofection. **(A)** Raw intracellular calcium flux data of [h-P2Y$_{12}$] (circle) and [Empty] (square) transfectants stimulated with exogenous ADP 10 µmol/L, representative of three experiments run in duplicate, red arrow indicates start of stimulation. **(B)** Baseline corrected peak calcium fluxes in response to stimulation of [h-P2Y$_{12}$] (circle) and [Empty] (square) with indicated concentrations of exogenous ADP, *n*=3 run in duplicate, n.s. not statistically significant, two-way ANOVA. Data represented as mean ± SEM. Relative fluorescence units (RFU).

3.3.3 Analysis of second messenger signalling of cysteinyl leukotriene stimulated human P2Y$_{12}$ transfectants

Many recent studies have well documented $CysLT_1$ receptors' abilities to activate both the $G\alpha_q$ and the $G\alpha_i$ signalling pathways. This promiscuity of an already known cysteinyl leukotriene receptor coupled with the fact the $G\alpha_i$ coupled receptors can induce calcium signalling led us to also analyse potential calcium signalling upon cysteinyl leukotriene stimulation in [h-$P2Y_{12}$] transfectants. On stimulation of both [Empty] and [h-$P2Y_{12}$] with varying concentrations of LTC_4, LTD_4 and LTE_4 (0.3-300 nmol/L), no differences in intracellular calcium mobilisation were observed between both transfectants (Figure 3.5A-C and Figure 3.6).

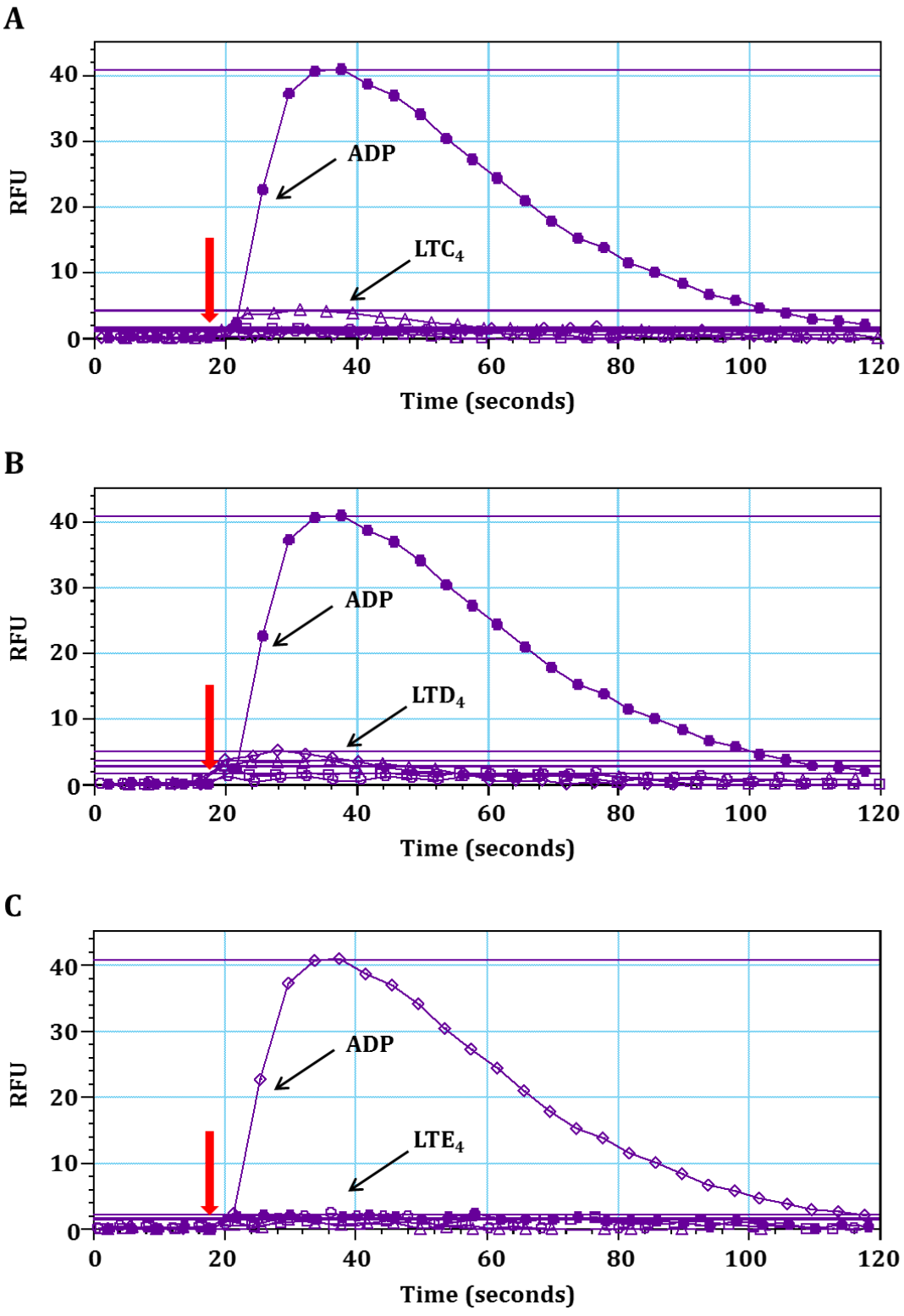


Figure 3.5 Cysteinyl leukotrienes do not induce calcium signalling in [h-P2Y$_{12}$] transfectants.

Forward constructs of 3xHA N-terminally tagged h-P2Y$_{12}$ in pcDNA3.1+ expression vectors were transiently transfected into separate HEK293T populations by lipofection. Raw intracellular calcium flux data of [h-P2Y$_{12}$] transfectants stimulated with a exogenous **(A)** LTC$_4$, 300 nmol/L (triangle), 30 nmol/L (square), 0.3 nmol/L (open circle) and 10 µmol/L

ADP (closed circle) and vehicle control (diamond), **(B)** LTD_4, 300 nmol/L (diamond), 30 nmol/L (triangle), 0.3 nmol/L (square) and 10 μmol/L ADP (closed circle) and vehicle control (open circle), **(C)** LTE_4, 300 nmol/L (open circle), 30 nmol/L (closed circle), 0.3 nmol/L (triangle) and ADP (diamond) 10 μmol/L, representative of three experiments run in duplicate, red arrow indicates start of stimulation. Relative fluorescence units (RFU).

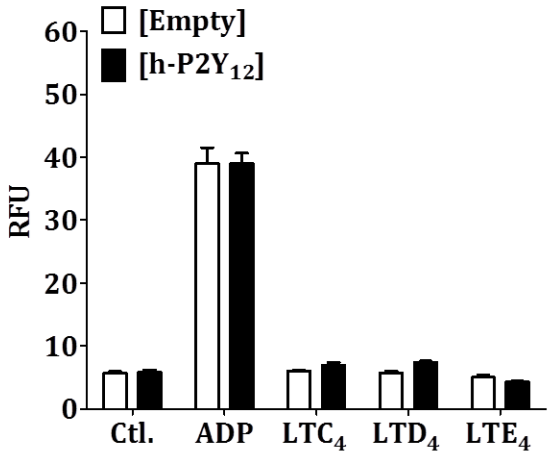


Figure 3.6 Cysteinyl leukotrienes do not induce calcium signalling in [h-$P2Y_{12}$] transfectants.

Forward constructs of 3xHA N-terminally tagged h-$P2Y_{12}$ and empty constructs in pcDNA3.1+ expression vectors were transiently transfected into separate HEK293T populations by lipofection. Peak calcium fluxes of [h-$P2Y_{12}$] (closed bars) and [Empty] (open bars) transfectants stimulated with either vehicle control, ADP 10 μmol/L, LTC_4 300 nmol/L, LTD_4 300 nmol/L or LTE_4 300 nmol/L, *n*=3 run in duplicate. Data represented as mean ± SEM. Relative fluorescence units (RFU).

3.3.4 Validation of functional co-overexpression of human P2Y$_{12}$ with Gα$_{16}$ in HEK293T cells

Gα$_{16}$ is a promiscuous Gα subunit that has been previously demonstrated to direct signal transduction of GPCRs to activate PLC when co-expressed (240). This technique of directing signalling transduction via PLC allows the analysis of GPCRs that signal through alternative signalling pathways. To demonstrate the effectiveness of this approach in our recombinant model, constructs containing human β_2 adrenergic (h-ADRβ_2) receptor, N-terminally 3xHA tagged were co-transfected with constructs containing h-Gα$_{16}$ into HEK293T cells and flow cytometry analysis verified that 66.18 ± 9 % of cells were considered positively expressing the receptor (mean ± SD, *n*=4, Figure 3.7A). On stimulation with isoproterenol, the natural ligand for ADRβ_2, there was a statistically significant increase ($p>0.001$; 2-way ANOVA) in intracellular calcium mobilisation in transfectants co-expressing h-Gα$_{16}$ compared to the single transfectant [h-ADRβ_2] and the empty vector control [Empty + Gα$_{16}$] (Figure 3.7B-C), therefore validating the susceptibility of GPCRs to signal modulation within our recombinant system.

Constructs containing N terminally 3xHA tagged h-P2Y$_{12}$ were then co-transfected with h-Gα$_{16}$ in HEK293T cells. qRT-PCR showed a significant increase in P2RY12 mRNA in the [h-P2Y$_{12}$ + Gα$_{16}$] transfectants compared to the [Empty + Gα$_{16}$] controls (Figure 3.7D). Analysis by flow cytometry confirmed that 71.32 ± 16.01 % of the cells were considered positively expressing P2Y$_{12}$ (mean ± SD, *n*=5, Figure 3.7A). These transfectants were able to show a statistically significant increase in intracellular calcium mobilisation to ADP ($p=0.022$; 2-way ANOVA) and its more stable derivative, 2-MeS-ADP ($p=0.019$; 2-way ANOVA), compared to the control transfectants (Figure 3.7E-F).

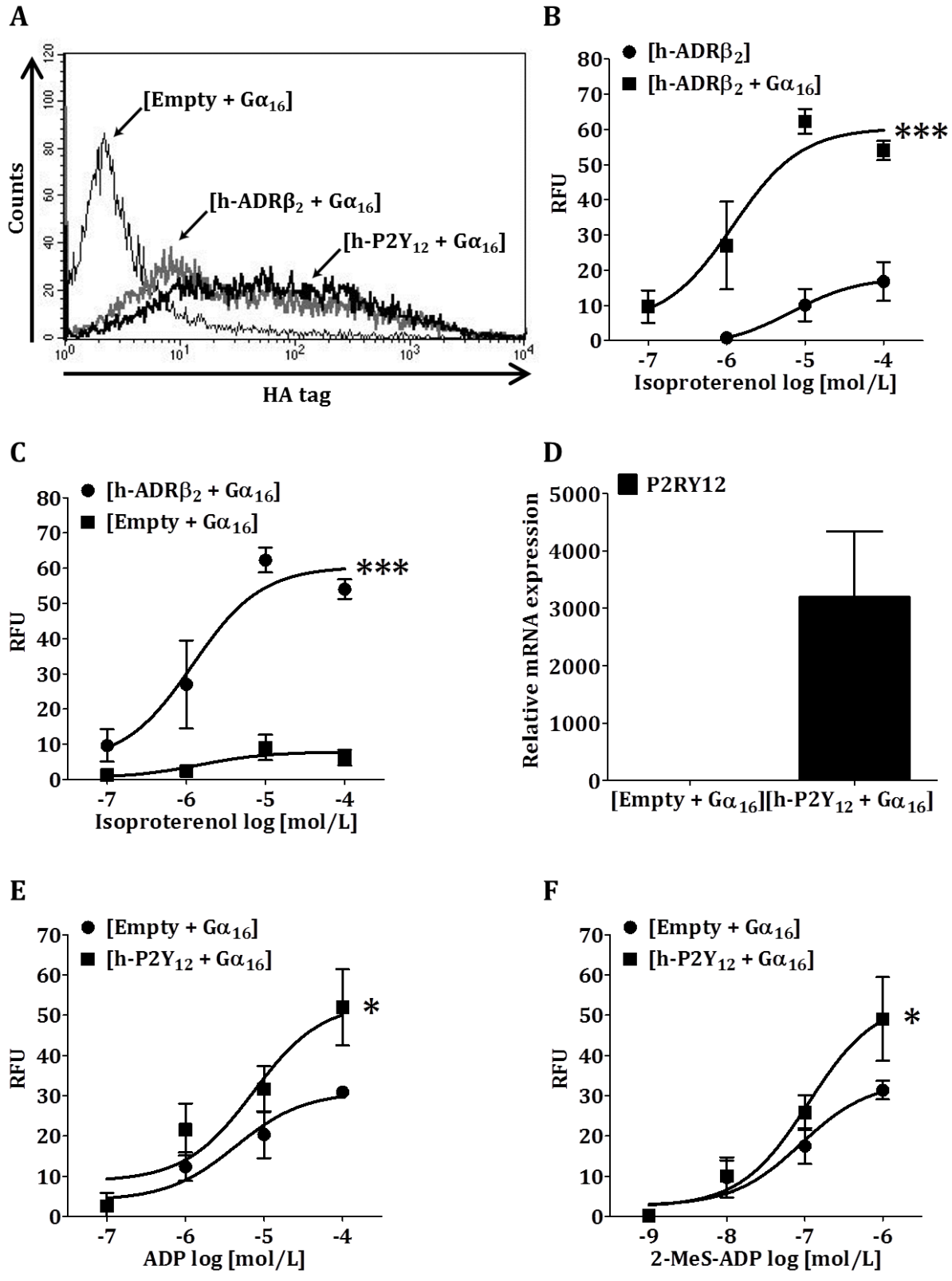


Figure 3.7 Validation of $G\alpha_{16}$ co-transfection model in HEK293T cells.

HEK293T cells were transiently transfected by lipofection with the indicated forward constructs in pcDNA3.1+ vectors. **(A)** Receptor expression analysis of 3xHA tagged h-$P2Y_{12}$ (bold black) and h-$ADR\beta_2$ (bold grey) co-expressed with $G\alpha_{16}$ compared to empty vector control (thin black) using an Alexa Fluor labelled antibody specific 3xHA, 1 in 1000,

results from a single experiment representative of 3. Baseline corrected peak calcium fluxes in response to stimulation with the indicated concentrations of isoproterenol of **(B)** [h-ADRβ_2] (circle), [h-ADRβ_2 + Gα_{16}] (square) and **(C)** [h-ADRβ_2 + Gα_{16}] (circle) and [Empty + Gα_{16}] (square), *n*=2-4. **(D)** Relative quantification of human P2RY12 mRNA in [h-P2Y$_{12}$ + Gα_{16}] compared to [Empty + Gα_{16}] control, 18s used as an endogenous control, *n*=3. Baseline corrected peak calcium fluxes in response to [h-P2Y$_{12}$ + Gα_{16}] and [Empty + Gα_{16}] transfectants stimulated with the indicated concentrations of exogenous **(E)** ADP and **(F)** 2-MeS-ADP, *n*=3. Data represented as mean ± SEM, two-way ANOVA, * $p<0.05$, *** $p<0.001$. Relative fluorescence units (RFU).

3.3.5 Analysis of second messenger signalling of cysteinyl leukotriene stimulated human P2Y$_{12}$ transfectants co-transfected with Gα_{16} in HEK293T

[h-P2Y$_{12}$ + Gα_{16}] transfectants were stimulated with varying concentrations (300-0.3 nmol/L) of cysteinyl leukotrienes and their effect on intracellular calcium mobilisation was analysed. As previously reported, ADP induced a robust flux in intracellular calcium (Figure 3.8A-C and Figure 3.9). All LTC$_4$, LTD$_4$ and LTE$_4$ stimulations induced calcium fluxes that were similar to the vehicle control, shown by the raw calcium flux data in Figure 3.8A-C and no statistically significant differences were observed in peak flux data in cysteinyl leukotriene responses between [h-P2Y$_{12}$ + Gα_{16}] and [Empty + Gα_{16}] transfectants (two-way ANOVA, Figure 3.9).

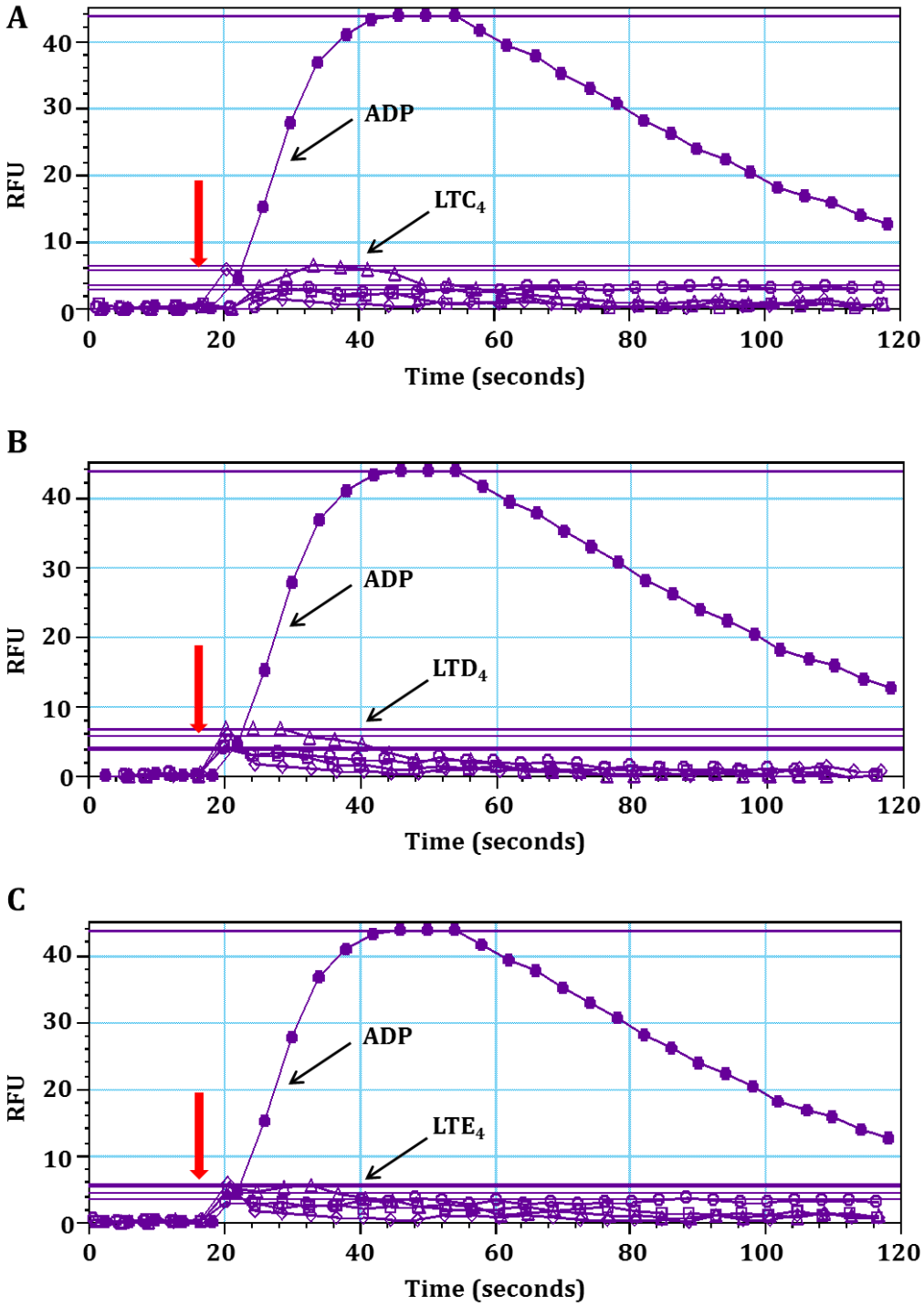


Figure 3.8 Cysteinyl leukotrienes do not induce intracellular calcium mobilisation in [h-P2Y12 + Gα16] transfectants.

Forward constructs of 3xHA N-terminally tagged h-$P2Y_{12}$ and empty constructs in pcDNA3.1+ expression vectors were co-transiently transfected with constructs containing $G\alpha_{16}$ into separate HEK293T populations by lipofection. Intracellular calcium fluxes of [h-$P2Y_{12}$ + $G\alpha_{16}$] transfectants stimulated with varying concentrations exogenous **(A)** LTC_4 **(B)** LTD_4 **(C)** LTE_4; 300 nmol/L (triangle), 30 nmol/L (square), 0.3 nmol/L (circle), each

with controls; ADP 10 μmol/L (closed circle) and vehicle control (diamond), representative of three experiments run in duplicate, red arrow indicates start of stimulation. Relative fluorescence units (RFU).

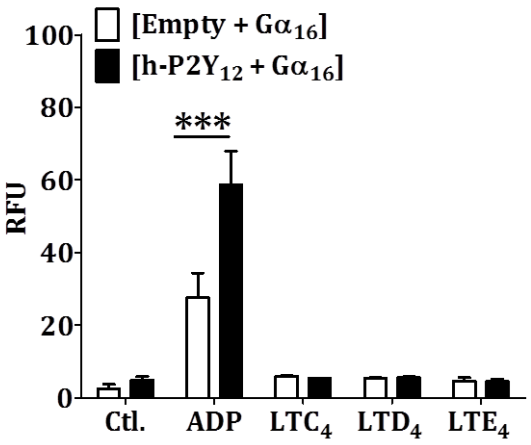


Figure 3.9 Cysteinyl leukotrienes do not induce intracellular calcium mobilisation in [h-P2Y12 + Gα16] transfectants.

Forward constructs of 3xHA N-terminally tagged h-$P2Y_{12}$ and empty constructs in pcDNA3.1+ expression vectors were co-transiently transfected with constructs containing $G\alpha_{16}$ into separate HEK293T populations by lipofection. Peak calcium fluxes of [h-$P2Y_{12}$ + $G\alpha_{16}$] (closed bars) and [Empty + $G\alpha_{16}$] (open bars) transfectants stimulated with either vehicle control, ADP 10 μmol/L, LTC_4 300 nmol/L, LTD_4 300 nmol/L or LTE_4 300 nmol/L, *n*=3 run in duplicate. Data represented as mean ± SEM, two-way ANOVA with Bonferroni post-hoc, *** $p<0.001$. Relative fluorescence units (RFU).

3.3.6 Analysis of alternative GPCR signalling pathways that could be activated by cysteinyl leukotrienes

Human $P2Y_{12}$ has been shown to signal physiologically through $G\alpha_i$ and by inhibition of intracellular cAMP generation. To analyse this potential signalling pathway, [h-$P2Y_{12}$] transfectants were stimulated with forskolin to activate adenylyl cyclase and to increase cAMP levels, together with either ADP or cysteinyl leukotrienes, intracellular cAMP was measured using a competitive immunoassay. ADP induced a significant, concentration-dependent inhibition of forskolin-induced cAMP in [h-$P2Y_{12}$] transfectants ($p=0.0048$, two-way ANOVA, Figure 3.10A). LTE_4 treatment showed no statistical difference in cAMP accumulation compared to the empty vector control ($p=0.81$, two-way ANOVA, Figure 3.10B) confirming that [h-$P2Y_{12}$] does not signal directly through the coupling of either $G\alpha_q$ or $G\alpha_i$ upon LTE_4 stimulation.

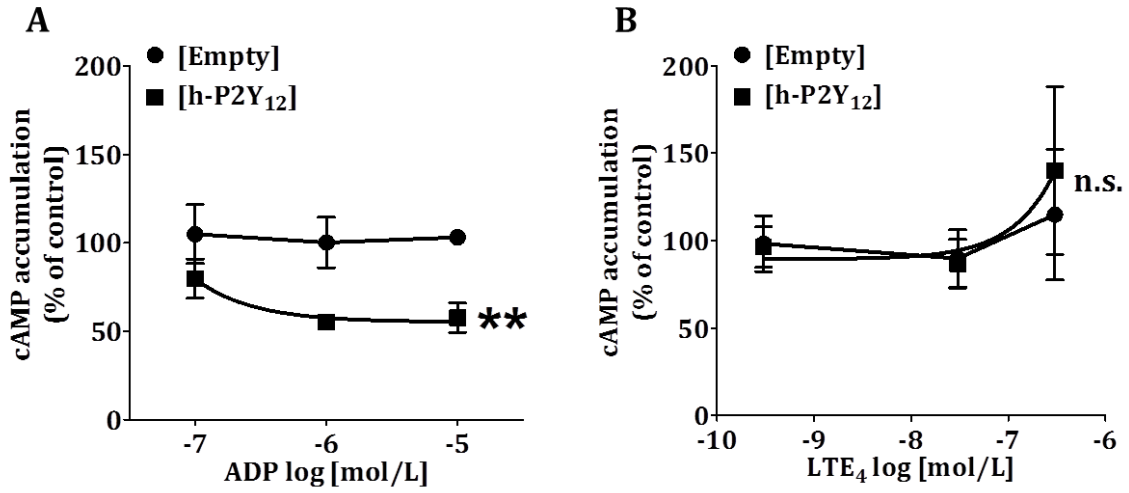


Figure 3.10 LTE_4 stimulation of [h-$P2Y_{12}$] does not activate $G\alpha_i$ signalling pathways.

Intracellular cAMP concentrations were analysed in models of HEK293T cells transiently transfected with forward constructs of 3xHA N-terminally tagged h-$P2Y_{12}$ and empty constructs in pcDNA3.1+ expression vectors. [h-$P2Y_{12}$] and [Empty] transfectants were stimulated with forskolin 10 μmol/L and the indicated concentration of either **(A)** ADP or **(B)** LTE_4, *n*=6, data expressed as % of forskolin stimulated control, mean ± SEM, two-way ANOVA, ** $p<0.01$.

Recruitment of β-arrestin is another activation event that can be analysed as a measure of GPCR activation. Until recently, an agonist's efficacy for β-arrestin recruitment was believed to be proportional to its efficacy for G-protein activities. However, it has been demonstrated that "biased ligands" can selectively activate β-arrestin function and elicit specific biological effects (241). To address the question of β-arrestin specific signalling induced by LTE_4, C-terminally modified h-$P2Y_{12}$ stably transfected into CHO cells with β-arrestin N-terminally tagged with a deletion mutant of β-galactosidase were stimulated with either 2-MeS-ADP or cysteinyl leukotrienes and processed according to manufacturer's protocols (Figure 3.11). Stimulation with 2-MeS-ADP induced a concentration-dependent luminescent signal relating to the recruitment of β-arrestin to the h-$P2Y_{12}$ receptor. Stimulation with LTE_4 and other cysteinyl leukotrienes (data not shown) showed no significant increase in signal suggesting a lack of β-arrestin pathway activation by leukotrienes. Collectively these observations show that cysteinyl leukotrienes do not induce G-protein-dependent or -independent signalling pathways directly through h-$P2Y_{12}$ indicating that h-$P2Y_{12}$ alone is not a cysteinyl leukotriene receptor.

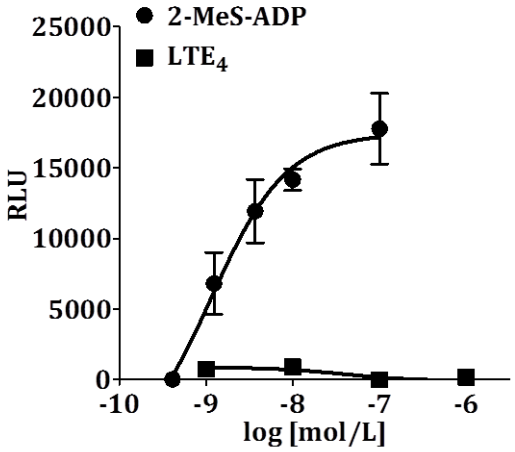


Figure 3.11 LTE_4 stimulation of [h-$P2Y_{12}$] does not activate β-arrestin recruitment.

β-arrestin recruitment was analysed in models of stably modified CHO cells with transfected with h-$P2Y_{12}$ and β-arrestin (DiscoverX). [h-$P2Y_{12}$] transfectants were stimulated with the indicated concentrations of either 2-MeS-ADP or LTE_4, *n*=3, expressed as relative luminescence units (RLU), mean ± SEM.

3.3.7 Analysis of second messenger signalling of cysteinyl leukotriene stimulated mouse $P2Y_{12}$ transfectants

The ability of LTE_4 to mediate pulmonary inflammation has been shown to be dependent on $P2Y_{12}$ expression in mouse models, as indicated by Paruchuri *et al.* (170). Strong evidence shows that on removal of the $P2Y_{12}$ receptor, either by knockout or by platelet depletion, the influx of inflammatory cells to the mouse lung can be significantly diminished. Although these findings are contradictory to our results, the lack of LTE_4 mediated signalling in out recombinant model could be due to species differences as mouse (m)-$P2Y_{12}$ shares only 89 % homology to its human derivative (Table 1.2) (120). To test this possibility a construct encoding m-$P2Y_{12}$ was transiently co-transfected with h-$G\alpha_{16}$ into HEK293T cells and stimulated with exogenous 2-MeS-ADP, LTC_4, LTD_4 or LTE_4. qRT-PCR confirmed that the transfection successfully incorporated mouse P2RY12 DNA (Figure 3.12A). 2-MeS-ADP stimulation of these co-transfections was able to induce a statistically significant increase in calcium mobilisation compared to the control transfectants (p=0.0101, two-way ANOVA, Figure 3.12B) indicating that h-$G\alpha_{16}$ is sufficiently able to direct the signalling pathway of m-$P2Y_{12}$, similar to that of h-$P2Y_{12}$ (Figure 3.12F). No specific calcium fluxes were observed on stimulation of these co-transfectants with LTE_4 (Figure 3.12C). These fluxes in calcium were similar to that of the vehicle control (open circle).

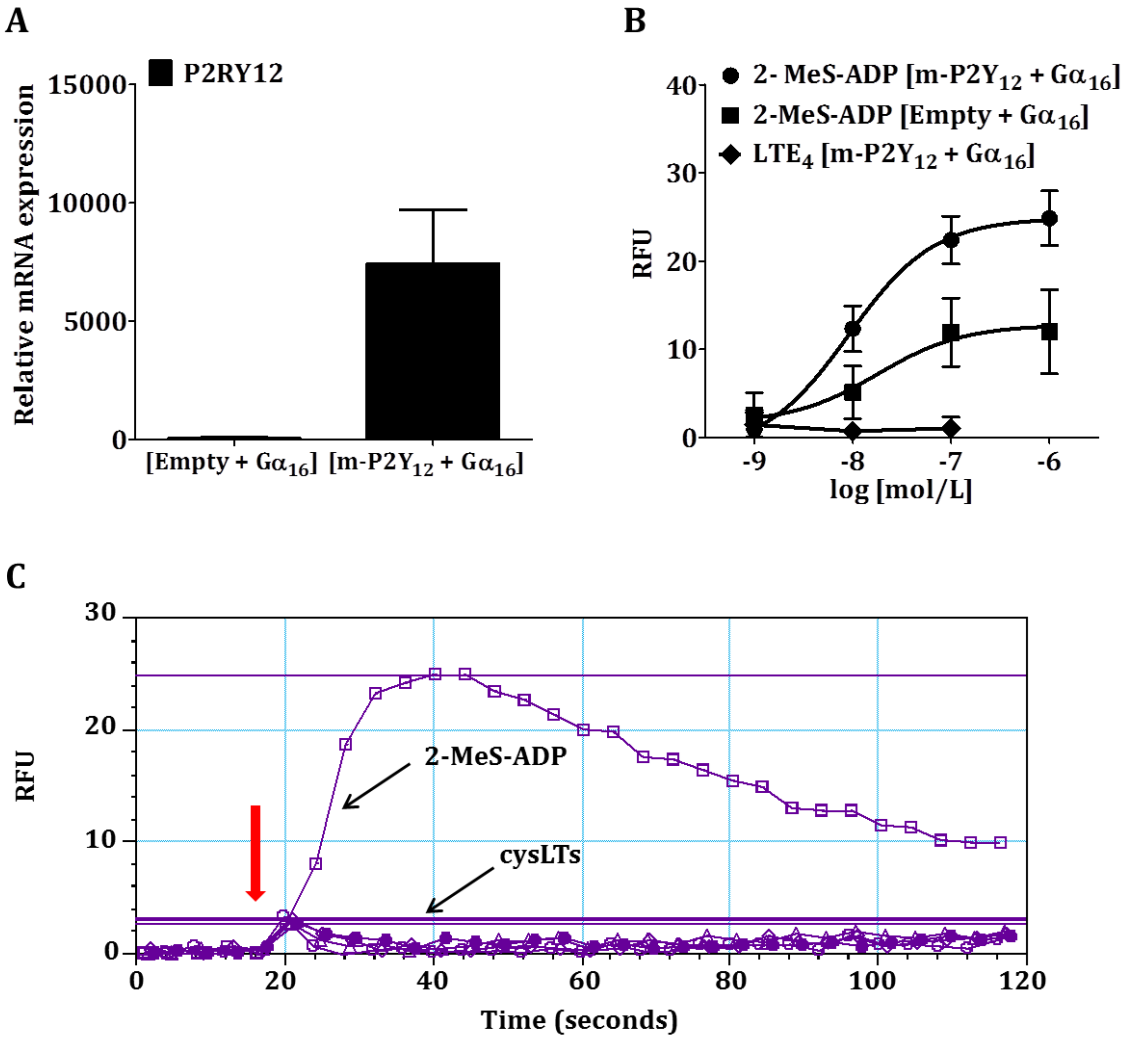


Figure 3.12 Cysteinyl leukotrienes do not induce calcium signalling in [m-P2Y12] transfectants.

Forward constructs of m-P2Y12 and empty constructs in pcDNA3.1+ expression vectors were co-transiently transfected with constructs containing $G\alpha_{16}$ into HEK293T cells by lipofection. **(A)** Relative quantification of mouse P2RY12 mRNA in [Empty + $G\alpha_{16}$] and [m-$P2Y_{12}$ + $G\alpha_{16}$] transfectants, respectively, compared with [Empty + $G\alpha_{16}$] transfectants, 18s used as an endogenous control, *n*=3. **(B)** Baseline corrected peak intracellular calcium fluxes were analysed in [m-$P2Y_{12}$ + $G\alpha_{16}$] (circle) and [Empty + $G\alpha_{16}$] (square) transfectants stimulated with the indicated concentrations of 2-MeS-ADP or LTE_4, *n*=3, mean ± SEM. **(C)** Raw intracellular calcium fluxes were analysed in [m-$P2Y_{12}$ + $G\alpha_{16}$] stimulated with 100 nmol/L of either LTE_4 (closed circle), LTD_4 (triangle), LTC_4 (diamond), 2-MeS-ADP 1 µmol/L (square) and vehicle control (open circle), representative of three experiments run in triplicate, cysteinyl leukotrienes (cysLTs), red arrow indicates start of stimulation. Relative fluorescence units (RFU).

3.3.8 Analysis of alternative signalling pathways in cysteinyl leukotriene stimulated mouse $P2Y_{12}$ transfectants

To determine whether LTE_4 could activate m-$P2Y_{12}$ via the recruitment of β-arrestin, stable CHO cell transfectants of C-terminally modified m-$P2Y_{12}$ and N-terminally tagged β-arrestin were stimulated with either 2-MeS-ADP or cysteinyl leukotrienes. Although stimulation with 2-MeS-ADP induced a dose-dependent luminescent signal, LTE_4 stimulation produced negligible effects (Figure 3.13A). These results show that m-$P2Y_{12}$ signalling responses are similar to those of h-$P2Y_{12}$.

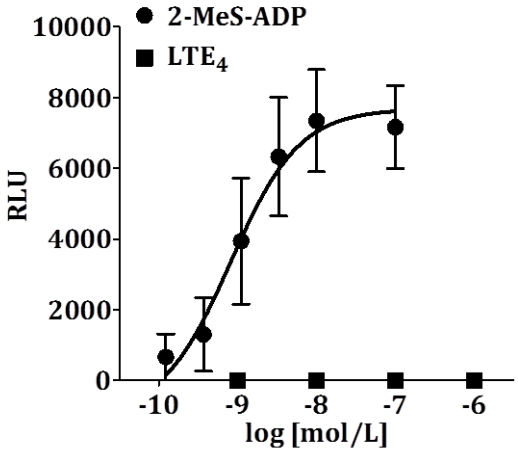


Figure 3.13 LTE_4 stimulation of [m-$P2Y_{12}$] does not activate β-arrestin recruitment.

β-arrestin recruitment was analysed in models of stably modified CHO cells transfected with m-$P2Y_{12}$ and β-arrestin (DiscoverX). [m-$P2Y_{12}$] transfectants were stimulated with the indicated concentrations of either 2-MeS-ADP or LTE_4, *n*=4, expressed as relative luminescence units (RLU), mean ± SEM.

3.3.9 Analysis of second messenger signalling in platelets stimulated with cysteinyl leukotrienes

Physiologically, the signalling capability of $P2Y_{12}$ is highly modulated by another ADP purinergic receptor, $P2Y_1$ (224). Signal modulation, whether this is through heterodimerisation or reciprocal cross-talk, allows the potentiation of signalling responses from both $P2Y_{12}$ and $P2Y_1$. To determine whether the importance of $P2Y_{12}$ in LTE_4 mediated pulmonary inflammation *in vivo* could be due to such interactions or heterodimerisation of GPCRs, isolated human platelets, one of very few cell types that highly expresses $P2Y_{12}$, were stimulated with 2-MeS-ADP and LTE_4 and their intracellular signalling responses were analysed. A robust dose-dependent intracellular calcium mobilisation and a statistically significant inhibition of forskolin-induced cAMP were generated by 2-MeS-ADP stimulation ($p<0.001$ one-way ANOVA, Figure 3.14A + Figure 3.15A) indicating that the platelets isolated from whole blood were functionally intact and are responsive to $P2Y_{12}$ agonists. Stimulation with LTE_4 induced no specific fluxes of intracellular calcium (Figure 3.14B-C). LTD_4 induced a dose-dependent trend, although not statistically significant, of inhibition of forskolin generated cAMP (Figure 3.15B) but LTE_4 was unable to induce any specific inhibition of cAMP (Figure 3.15C).

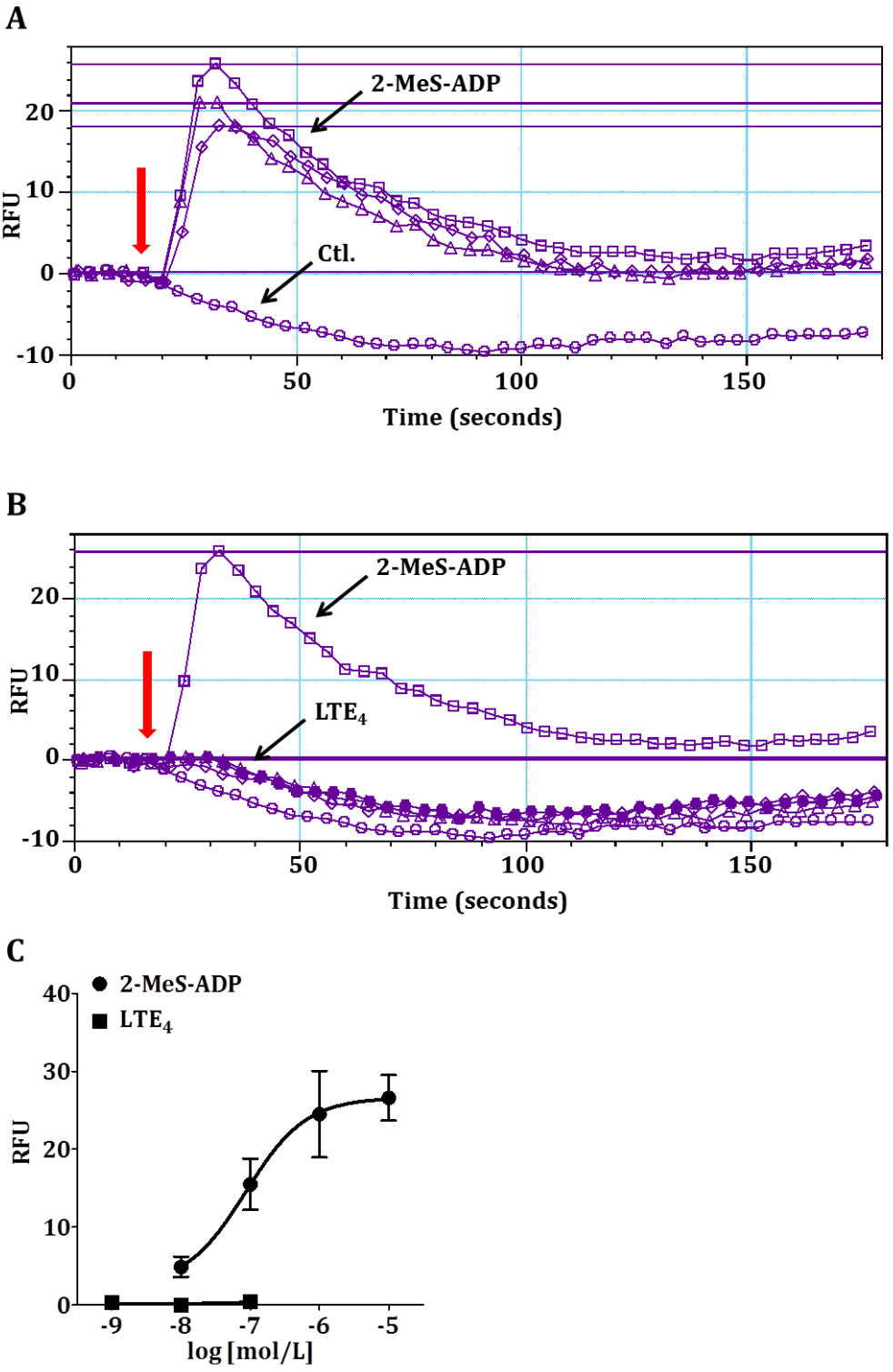


Figure 3.14 Cysteinyl leukotrienes do not induce intracellular calcium mobilisation within human platelets.

Human platelets isolated from peripheral blood were analysed for second messenger signalling responses upon stimulation. Raw calcium flux data of platelets stimulated with **(A)** 2-MeS-ADP; 10 µmol/L (square), 1 µmol/L (triangle), 0.1 µmol/L (diamond) and vehicle control (Ctl., circle), representative of 3 donors run in at least duplicate, **(B)** LTE_4; 100 nmol/L (triangle), 10 nmol/L (diamond), 1 nmol/L (closed circle), 2-MeS-ADP 10

µmol/L (circle) and vehicle control (open circle), representative of 3 donors run in at least duplicate. **(C)** Baseline corrected peak intracellular calcium fluxes of platelets stimulated with the indicated concentrations of 2-MeS-ADP or LTE_4, data from 3 donors run in duplicate, mean ± SEM, red arrow indicates start of stimulation. Relative fluorescence units (RFU).

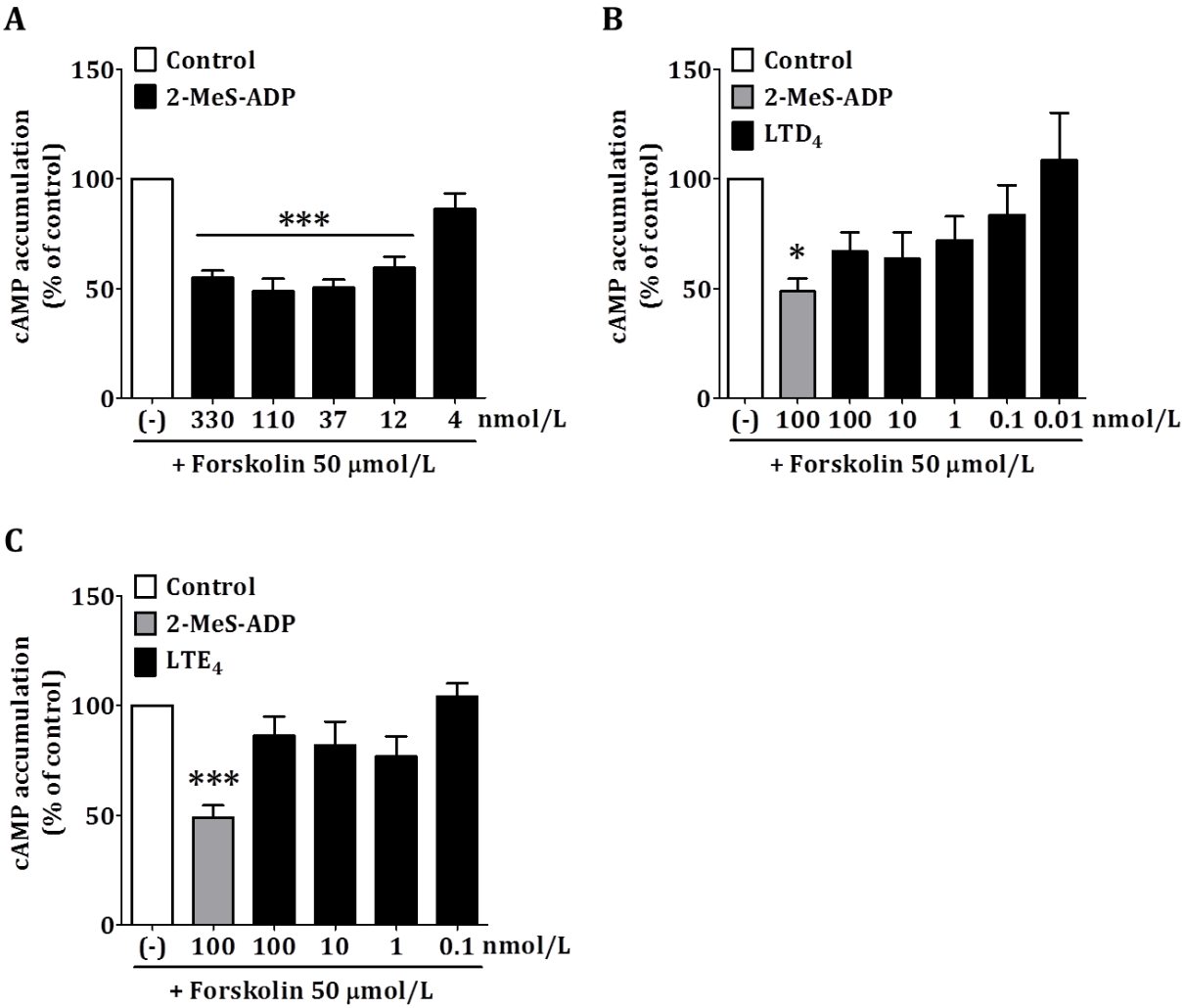


Figure 3.15 Cysteinyl leukotrienes do not induce the inhibition of intracellular cAMP within human platelets.

Human platelets isolated from peripheral blood were stimulated with forskolin 50 µmol/L and with the indicated concentrations of **(A)** 2-MeS-ADP, data from 5 donors run in triplicate, **(B)** 2-MeS-ADP or LTD_4, data from 3-5 donors run in triplicate, **(C)** 2-MeS-ADP or LTE_4, data from 3-6 donors run in triplicate. Data expressed as % of forskolin stimulated control, mean ± SEM, one-way ANOVA with Dunnetts post hoc, * $p<0.05$, *** $p<0.001$.

3.3.10 Analysis of platelet activation by cysteinyl leukotrienes

Activated platelets enter into an aggregation cascade where adhesion molecules are upregulated on the cell surface and a range of stored mediators are released to enhance this process. To address the question of whether cysteinyl leukotrienes are able to activate platelets through $P2Y_{12}$ or physiologically expressed receptor heterodimers, platelet activation was measured by expression of P-selectin (CD62P) and release of the stored chemokine CCL5 (RANTES). Whole blood was stimulated with either ADP, 2-MeS-ADP or cysteinyl leukotrienes and CD62P expression was measured by flow cytometry on the $CD61^+$ population of human platelets (Figure 3.16A). ADP and 2-MeS-ADP induced a statistically significant increase in $CD62P^+$ in platelets ($p<0.001$, two-way ANOVA, Figure 3.16B) while LTC_4, LTD_4 or LTE_4 were unable to induce any specific effects. Similarly, human platelets stimulated with calcium ionophore or ADP released increased amounts of CCL5 but no such response was identified upon stimulation with LTC_4, LTD_4 or LTE_4 (Figure 3.17A-C).

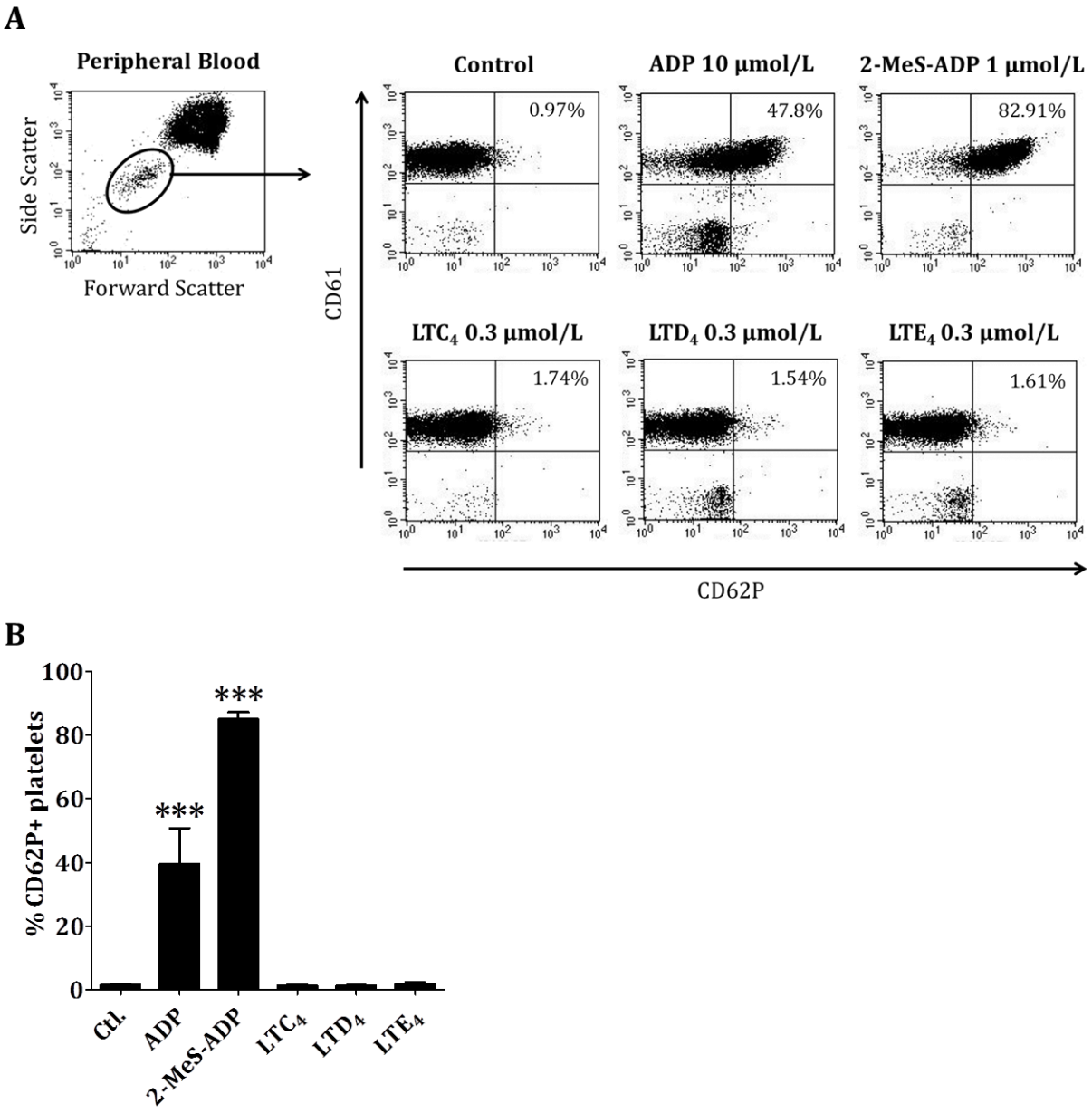


Figure 3.16 Cysteinyl leukotrienes do not upregulate the platelet activation marker CD62P.

Whole blood stimulated with either ADP 10 µmol/L, 2-MeS-ADP 1 µmol/L, LTC_4 0.3 µmol/L, LTD_4 0.3 µmol/L or LTE_4 0.3 µmol/L for 10 minutes was stained with monoclonal antibodies for CD61 and CD62P. **(A-B)** Flow cytometric analysis of platelet activation was performed on the gated platelet population shown in the oval gate on forward and side scatter dot plot of peripheral blood, **(A)** Dot plot representative of 3 experiments with different donors, **(B)** cumulative mean ± SEM of % $CD62P^+$ platelets, 3 experiments with different donors, one-way ANOVA with Dunnetts post-hoc, *** $p<0.001$.

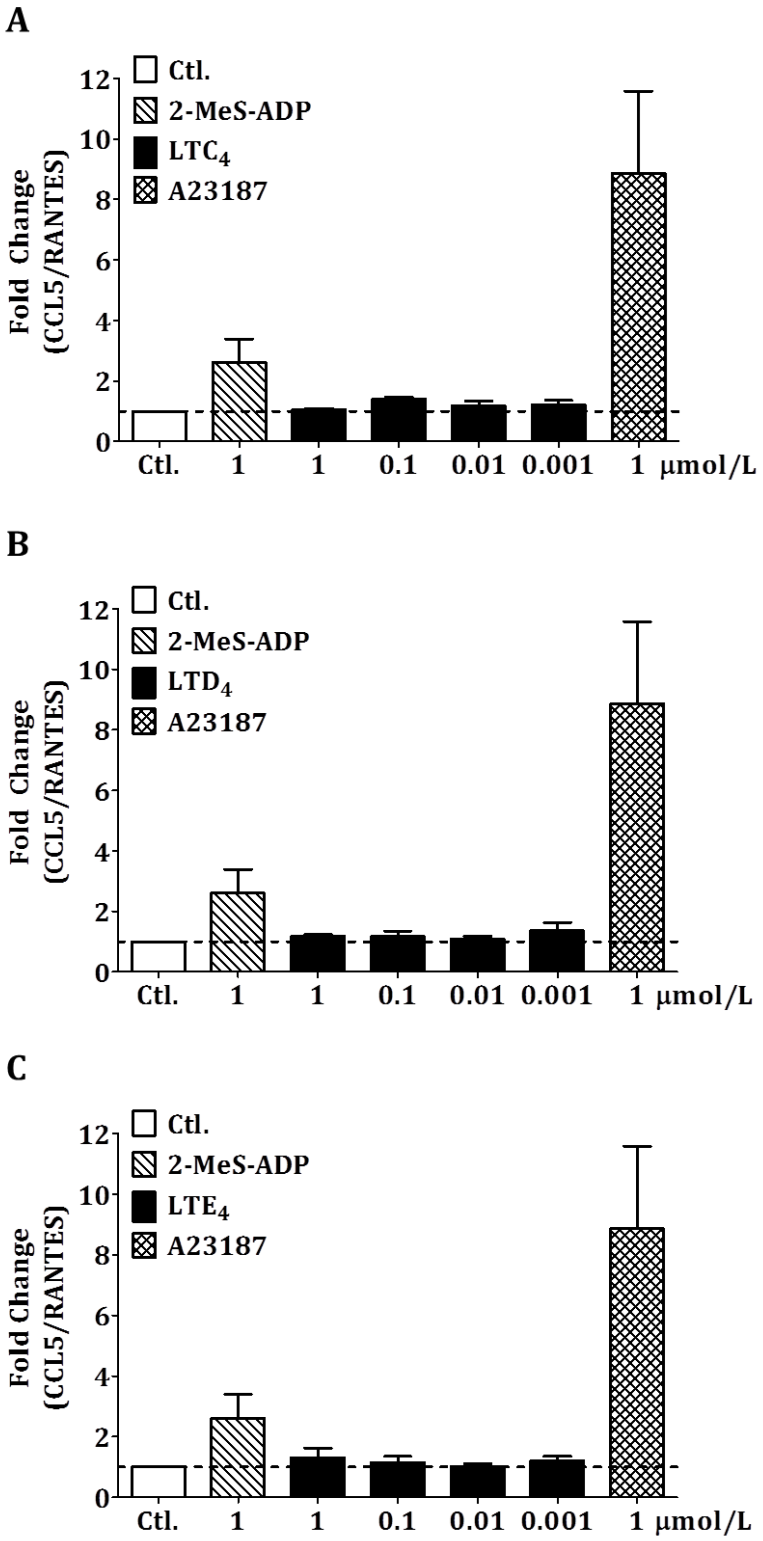


Figure 3.17 Cysteinyl leukotrienes do not induce the release of CCL5 from human platelets.

Human platelets isolated from peripheral blood were stimulated and their supernatants collected for protein analysis. **(A-C)** CCL5 concentrations were analysed by ELISA of supernatants collected from platelets stimulated with the indicated concentrations of **(A)** LTC_4, **(B)** LTD_4, **(C)** LTE_4, and vehicle control (Ctl.), 2-MeS-ADP, calcium ionophore

A23187, data expressed as fold change compared to the vehicle control, mean ± SEM of 3 experiments with different donors run in duplicate.

3.3.11 Analysis of OXGR1 as a potential third cysteinyl leukotriene receptor

To ascertain whether LTE_4 could mediate signal transduction through the OXGR1 receptor, heterologous expression models were generated and second messenger signalling was analysed. As OXGR1 has previously been identified as a $G\alpha_q$ coupled receptor when stimulated with α-ketoglutarate, HEK293T cells transiently transfected with constructs containing human OXGR1 were stimulated with α-ketoglutarate and intracellular calcium mobilisation was analysed (232). Native HEK293T cells did not respond to α-ketoglutarate stimulation. [h-OXGR1] transfectants stimulated with α-ketoglutarate generated a dose-dependent flux in intracellular calcium mobilisation confirming a functional overexpression of this receptor (Figure 3.18A). Cysteinyl leukotriene stimulation of these transfectants using varying concentrations (1-100 nmol/L) did not produce any specific fluxes in intracellular calcium mobilisation (Figure 3.18B). To make sure cysteinyl leukotrienes were not activating any other signalling pathways, OXGR1 was co-transfected with h-$G\alpha_{16}$ into HEK293T cells. [h-OXGR1 + $G\alpha_{16}$] transfectants stimulated with α-ketoglutarate again produced a dose-dependent flux in intracellular calcium mobilisation which was of similar intensity to the single transfectant model (Figure 3.18C). Varying concentrations of cysteinyl leukotrienes did not induce mobilisation of intracellular calcium (Figure 3.18D).

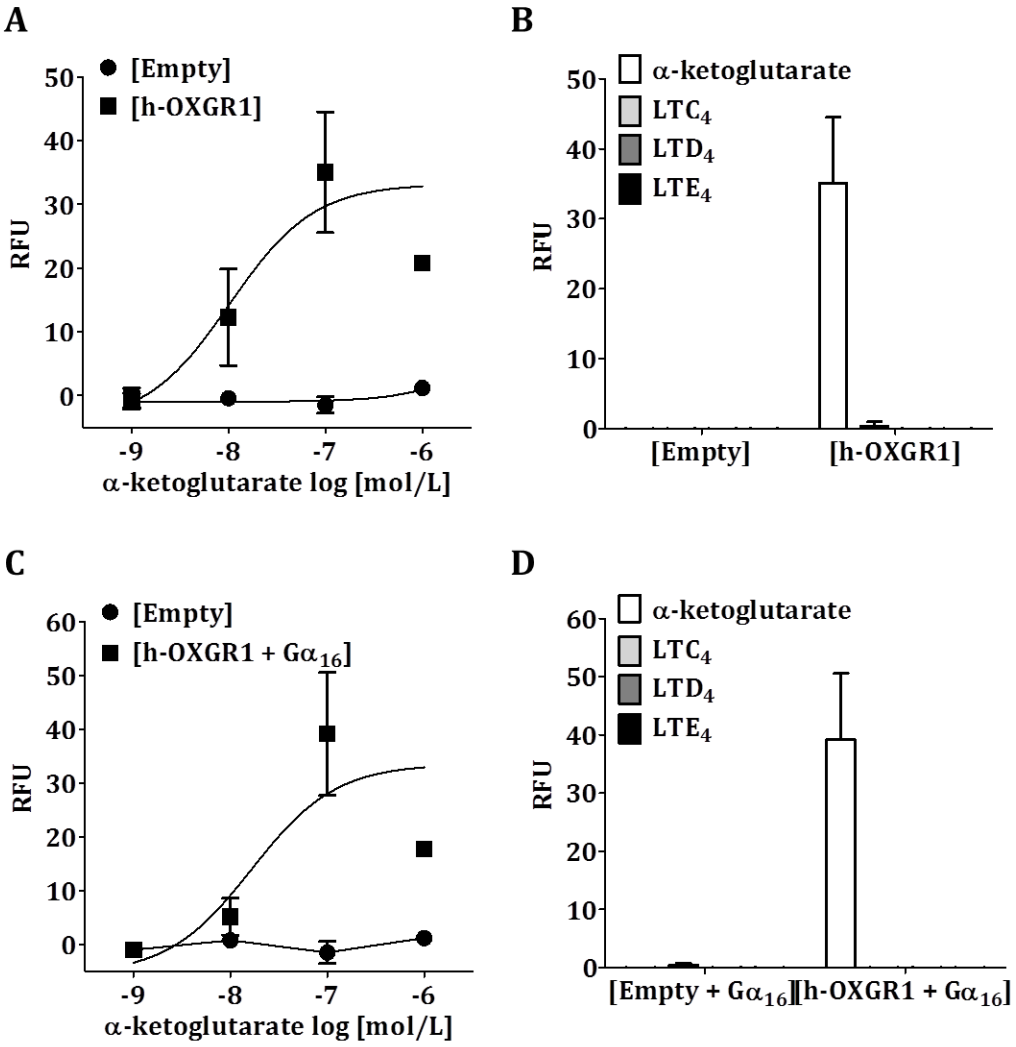


Figure 3.18 Cysteinyl leukotrienes do not induce signalling through human OXGR1. HEK293T cells were transfected with forward constructs of **(A-B)** h-OXGR1 (GPR99) or **(C-D)** co-transfected with h-OXGR1 and $G\alpha_{16}$ using lipofection and were analysed for intracellular calcium mobilisation upon cysteinyl leukotriene stimulation. Baseline corrected peak intracellular calcium flux data of **(A + C)** transfectants and the empty vector control stimulated with the indicated concentrations of α-ketoglutarate, *n*=1-3 run in triplicates, **(B + D)** transfectants and the empty vector control stimulated with the indicated concentrations of α-ketoglutarate 100 nmol/L, LTC_4 100 nmol/L, LTD_4 100 nmol/L and LTE_4 100 nmol/L, *n*=3 run in triplicates, mean ± SEM. Relative fluorescence units (RFU).

3.4 Discussion

Recent studies have suggested that LTE_4's potent activity does not involve activation of any of the classical cysteinyl leukotriene receptors, i.e. $CysLT_1$ or $CysLT_2$, but rather the activation of a third novel cysteinyl leukotriene receptor, "$CysLT_3$". Low dose OVA-induced allergic lung inflammation in mice lacking $P2Y_{12}$ functionality either by knockdown, platelet depletion or receptor antagonism, exhibited a substantial reduction in LTE_4 mediated pulmonary inflammation, a phenomenon not observed in $CysLT_1/CysLT_2$ double knockout mice (170). More recently, triple knockout mice $OXGR1/CysLT_1/CysLT_2$ have been shown to have a substantial abrogation of LTE_4-induced ear oedema compared to the wild type suggesting that OXGR1 could be another potential LTE_4 receptor (236). This study was undertook to determine whether these observations were specifically related to direct receptor-LTE_4 interactions, therefore determining whether $P2Y_{12}$ or OXGR1 are in fact cysteinyl leukotriene receptors.

Firstly, a heterologous expression model was established in which the recombinant receptor of interest could be transiently overexpressed in HEK293T cells. The best understood cysteinyl leukotriene receptor signalling pathway couples to $G\alpha_q$; thus, functional validation of the expression model was carried out by analysing intracellular calcium mobilisation. The pattern of ligand efficacies in both [$CysLT_1$] and [$CysLT_2$] transfectants matched those observed in the literature, LTD_4 being the most potent agonist and LTE_4 being the weakest (238,239). The receptor responses were also susceptible to their selective antagonists ($CysLT_1$ antagonists; MK-571 and Montelukast and $CysLT_2$ antagonist; HAMI 3379), therefore validating the expression model (Figure 3.2). Unsurprisingly, calcium mobilisation was not induced by cysteinyl leukotriene stimulation of [$P2Y_{12}$] transfectants, as the purinergic receptor has been well characterised as a GPCR that mainly activates $G\alpha_i$ signalling pathways, affecting intracellular cAMP levels. However, the lack of LTE_4 calcium mobilisation in [$P2Y_{12}$] transfectants co-expressing the $G\alpha_{16}$ protein was in stark contrast with the initial study by Nonaka *et al.* identifying LTE_4 as a surrogate ligand for $P2Y_{12}$ (233). They showed that LTE_4, but not LTC_4 or LTD_4, was able to induce intracellular calcium flux in CHO cells stably overexpressing both $P2Y_{12}$ and $G\alpha_{16}$. As different platforms for the recombinant model were utilised, as well as different transfection techniques, this could be a reason why different signalling responses were observed. Further confirmation of our observations was shown by the negligible effect of LTE_4 and other cysteinyl leukotrienes on cAMP accumulation and β-arrestin recruitment (Figure 3.10, Figure 3.11), two intracellular signalling pathways being potently activated in the same assays by known $P2Y_{12}$ agonists, ADP and 2-MeS-ADP. This lack of LTE_4-induced signalling was mimicked in transfectants

containing the mouse version of the $P2Y_{12}$ receptor (Figure 3.12, Figure 3.13) suggesting that the deficiency in signalling responses was not merely a human phenomenon and was independent of species variation. As no direct $P2Y_{12}$ signalling upon cysteinyl leukotriene stimulation was observed in any of our recombinant experiments, the possibility that LTE_4 could activate cells through another GPCR forming a heterodimer with $P2Y_{12}$ *in vivo* was addressed. Platelets are one of very few cell types that functionally express $P2Y_{12}$ and platelet depletion potently inhibited LTE_4-mediated pulmonary inflammation so human platelets were used to verify whether those cells are able to respond to cysteinyl leukotrienes (170). No specific responses to LTE_4 or other leukotrienes were observed when intracellular signalling (calcium, cAMP) as well as cell activation (P-selectin expression and CCL5 release) was measured. In contrast, human platelets strongly responded to known $P2Y_{12}$ agonists and non-specific activators in those assays showing that the cells were able to respond to appropriate stimulations implying that platelets are not a direct target for leukotrienes.

If platelets and $P2Y_{12}$ do not respond to LTE_4 as our data suggest, a question arises of how observations from Paruchuri *et al.* on LTE_4 mediated pulmonary inflammation may be explained? Our hypothesis is that LTE_4 must activate specific receptors present on cells other than platelets, potentially structural cells such as endothelial cells, smooth muscle cells or tissue resident cells i.e. mast cells. Upon LTE_4 activation, such cells would produce (release) mediators that activate platelets or platelet-adhering leukocytes, facilitating cell adhesion to endothelium, cell activation and migration to tissue and as a result enhancing inflammatory responses. Platelet involvement in proinflammatory reactions, especially in pulmonary inflammation observed in asthma has been of increased interest recently. Clinical evidence has demonstrated increases in circulating platelets in atopic asthmatics, as well as increases in leukocyte-platelet aggregates after allergen challenge (242-244). Recent advancements in the field have shown the direct importance of platelets in leukocyte recruitment and airway remodelling in allergic inflammation (170,245-248), therefore the reduction in LTE_4-mediated pulmonary inflammation seen in the study by Paruchuri *et al.* could be directly due to loss of $P2Y_{12}$ functionality rather than an LTE_4 specific phenomenon (170).

As OXGR1 had also been potentially identified as a third cysteinyl leukotriene receptor in mouse models of inflammation, second messenger signalling events in [h-OXGR1] transfectants were analysed(236). Using the same heterologous expression model as the $P2Y_{12}$ study, human OXGR1 overexpressed in HEK293T cells showed robust intracellular calcium mobilisation to its known ligand, α-ketoglutarate, indicating a functional overexpression of this receptor (Figure 3.18). However, cysteinyl leukotrienes did not

induce any specific fluxes in intracellular calcium even in the presence of $G\alpha_{16}$, strongly indicating that cysteinyl leukotrienes do not directly activate this receptor.

Cysteinyl leukotrienes can induce vascular permeability but the mechanism of their action has not been fully elucidated. Maekawa *et al*.'s study shows that cysteinyl leukotrienes can induce a local vascular reaction that does not involve cellular infiltration into the tissue (237). Curiously it was only the knockdown of all three receptors $CysLT_1$, $CysLT_2$ and OXGR1 that enabled significant inhibition of vascular oedema (236). As OXGR1 has been shown not to be a cysteinyl leukotriene receptor this would suggest that potentially all three receptors are involved in this mechanism, with OXGR1 not acting as a direct target for leukotrienes but being rather indirectly involved in leukotriene-induced inflammation. Unfortunately the role of α-ketoglutarate, the natural ligand for OXGR1, in vascular permeability does not support the data presented by Kanaoka *et al*.. α-Ketoglutarate is a major component in the Krebs cycle and has been shown to modulate cell cycle by increasing the levels of cyclin-cell-dependent kinase inhibitors and can also act as a substrate for the degradation of the hypoxia-inducible factor 1, which is a key protein in regulating responses to hypoxia (249-251). α-Ketoglutarate is thought to have anti-tumour properties and can also suppress vascular endothelial growth factor (VEGF) transcription and release (249). VEGF is a major angiogenetic factor involved in endothelial cell proliferation, tubule formation and increased microvascular permeability. However, whether any of these observations were receptor mediated is debatable and so the identification of OXGR1's involvement in cysteinyl leukotriene-induced vascular oedema could suggest a new role for α-ketoglutarate/OXGR1 which needs further investigation.

Interestingly, both knockout mouse models provide strong evidence for LTE_4-induced responses mediated independently of the classical cysteinyl leukotriene receptors, $CysLT_1$ and $CysLT_2$ (170,237) showing that LTE_4 may preferentially signal via another, as yet unidentified, cysteinyl leukotriene receptor.

Chapter 4

Identification of the GPCR responsible for preferential LTE_4 signalling within human mast cells

4.1 Introduction

Mast cells are derived from hematopoietic stem cells that originate from bone marrow. These $CD34^+$ and $CD117^+$ ($c\text{-}kit^+$) progenitors are released into the circulation where on specific cues they can migrate into the tissue. Maturation into terminal mast cells is thought to only occur in the tissue in response to specific mediators and cytokines that are released into the tissue moiety. It is mostly dependent on c-kit activation which is a receptor tyrosine kinase that binds stem cell factor (SCF) and is essential for mast cell growth. c-kit activation can occur in two ways; SCF-induced c-kit dimerization or auto-phosphorylation (252,253). Mast cells are thought to be pluripotent cells unique in the fact that on activation with an appropriate ligand, they are able to release a magnitude of pro-inflammatory mediators by a process known as degranulation, when cytoplasmic granules, rich in amines, glycoproteins and proteases are exocytosed into the extracellular space. Mast cells are also able to release newly generated mediators, such as metabolites of arachidonic acid (prostaglandins and leukotrienes) as well as various cytokines and chemokines. The most clinically relevant of these are histamine, prostaglandins and leukotrienes which contribute to the asthmatic phenotype such as induction of bronchial constriction and chemotaxis of inflammatory cells (eosinophils, Th2 cells and basophils). Mast cells have been shown to express both functional $CysLT_1$ and $CysLT_2$ receptors that leads to an interesting regulatory pathway of cysteinyl leukotrienes acting in both a para- and autocrine fashion (125,140,254).

Research using human mast cells is difficult due to the scarce nature of these cells in tissue and the complex isolation processes. Cells differentiated *in vitro* from cord/peripheral blood do offer an alternative approach but these cells are difficult to culture and are not fully representative of tissue-derived cells. There are currently three human mast cell lines available that have spontaneously arisen from $CD34^+$ cultures; HMC-1, LAD2 and the recently developed LUVA cells (19,219,255). Both HMC-1 and LAD2 cells are derived from patients with mast cell leukaemia but LUVA cells are the first mast cell line derived from a patient without any mast cell abnormalities. On comparison to skin mast cells, HMC-1 and LAD2 cells were found to express many mast cell markers (e.g. FcεRI, c-kit) although HMC-1 has a mutated form of c-kit that causes auto-phosphorylation and so does not require SCF for proliferation (255,256). HMC-1 cells were also found to have very low levels of FcεRI and on IgE cross–linking they were not able to release any detectable levels of histamine (256). LAD2 and skin mast cells had comparable levels of FcεRI and spontaneous release of histamine was only slightly reduced in LAD2 cells. The major differences seen in these two cell lines compared with skin mast cells was the significantly reduced levels of tryptase and chymase in HMC-1 and LAD2 cells. It is thought that these

two enzymes are produced late in mast cell differentiation and thus HMC-1 is considered a very immature mast cell line while LAD2 cells are believed to represent more mature mast cell phenotype (intermediate level of differentiation) (256).

LUVA cells are a relatively new mast cell line that has been shown to express both c-kit and FcεRI. The c-kit receptor in LUVA cells is not mutated, it is phosphorylated at very low level at baseline and cells respond to the c-kit ligand (SCF) with accelerated proliferation. LUVA cells do not require SCF for proliferation which suggests a c-kit-independent pathway that is currently unknown (19). Immunochemical staining and transcript expression show that LUVA cells express both tryptase and chymase, similarly to LAD2 and CD34+ derived primary mast cells, suggesting LUVA cells to be another partially mature mast cell line.

Jiang *et al*. first highlighted the role of cysteinyl leukotrienes in inducing co- mitogenesis of primary human mast cells derived from cord blood with the growth factor SCF (254). LTE_4 was the most potent of the three cysteinyl leukotrienes for inducing mast cell proliferation, an effect that was attenuated by the use of the selective $CysLT_1$ antagonist, MK-571. These observations of LTE_4's potency were repeated in LAD2 cells (19,257). LTE_4 was more potent than LTD_4 at inducing proliferative signals, mimicking Jiang *et al.*'s original paper and regulated cytokine and chemokine mRNA expression (IL-5, IL-8, TNF-α, CCL4 and CCL2), either greater than or equal to that of LTD_4 (254,257). CCL4 and prostaglandin D_2 were both measurable in the supernatants of LAD2 cells with again LTE_4 being the most potent cysteinyl leukotriene. All responses were sensitive to $CysLT_1$ antagonism by MK-571 but LTE_4's response was relatively unaffected by knockdown of $CysLT_1$ using short-hairpin RNA (shRNA) suggesting the involvement of a MK-571 sensitive GPCR other than $CysLT_1$ (257).

LTD_4 and LTE_4's ability to robustly regulate gene expression in human mast cell lines has previously been analysed in our group (unpublished data). Microarray analysis of LAD2 cells stimulated with either vehicle control, LTD_4 (100 nmol/L) or LTE_4 (100 nmol/L) for 2 hours was carried out in the presence of L-cysteine (inhibits the dipeptidase enzyme that converts LTD_4 to LTE_4). The heat map representation of the hierarchal clustering of the array data reveals how extensively cysteinyl leukotrienes regulate gene expression in LAD2 cells (Figure 4.1, Table 4.1). LTD_4 robustly up and down regulates genes within LAD2 cells and LTE_4 in many instances is more potent at doing this than LTD_4. The genes most highly upregulated by LTE_4 are a multitude of chemokines, growth and transcriptional factors that are also upregulated by LTD_4. Unsurprisingly, CCL4 is one of the most highly upregulated genes by both LTE_4 and LTD_4. It is also the highest

differentially expressed gene when comparing responses to LTE_4 and LTD_4 (8.3 fold change), which is supportive of data already published in the literature (170,257).

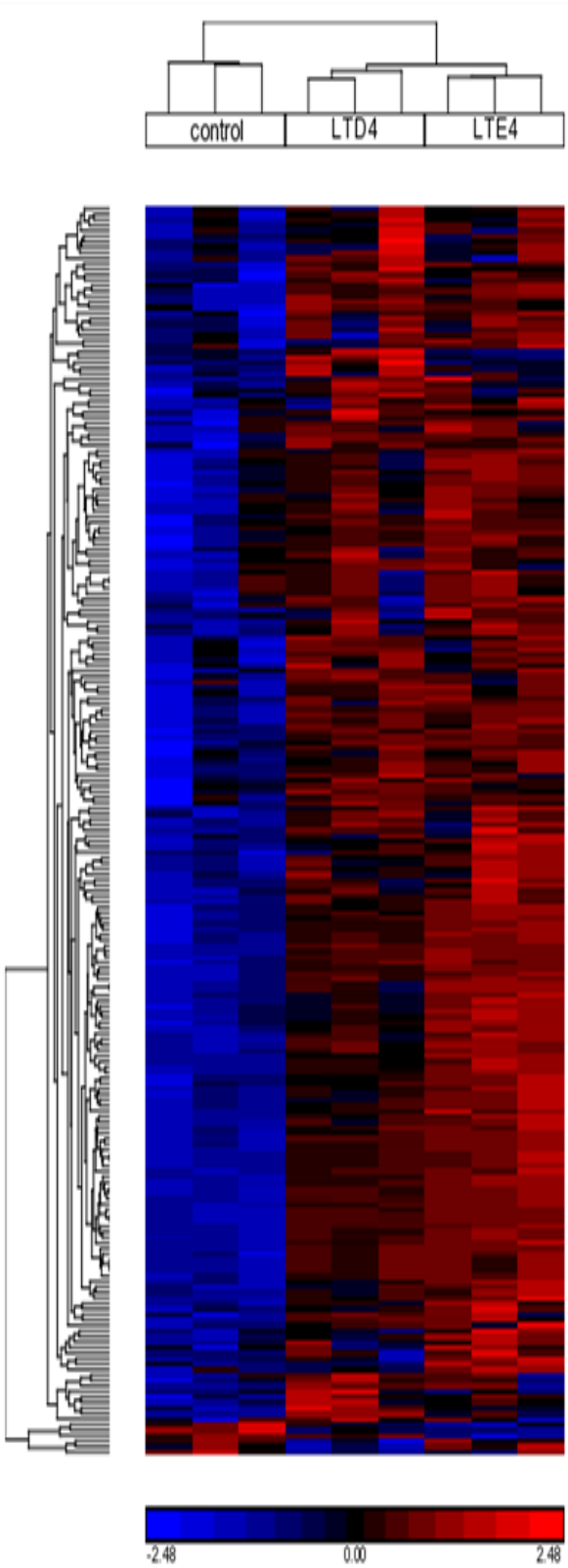


Figure 4.1 Microarray analysis of cysteinyl leukotriene stimulated LAD2 cells.

LAD2 cells (*n*=3) were stimulated with vehicle control, LTD_4 or LTE_4 (100 nM) for 2 hours and gene expression was analysed using Affymetrix Human Gene 1.0 ST microarrays.

Table 4.1 List of the most differentially expressed genes in LAD2 cells stimulated with cysteinyl leukotrienes.

Gene	Description	Control	LTE_4		LTD_4		Fold Change
		RI	RI	Fold Change (vs. control)	RI	Fold Change (vs. control)	LTE_4 vs. LTD_4
CCL4L1	Chemokine ligand 4-like 1	7.94	12.32	20.88	10.09	4.44	4.70
CCL4	Chemokine ligand 4	6.20	10.51	19.95	7.46	2.40	8.30
EGR3	Early growth response 3	7.02	11.29	19.34	9.27	4.76	4.07
STATH	Statherin	4.85	7.61	6.76	6.09	2.36	2.87
PHLDA1	Pleckstrin homology-like domain, family A, member 1	7.05	9.70	6.28	9.07	4.06	1.55
NFKBID	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, delta	8.38	10.99	6.09	9.88	2.82	2.16
BCL6	B-cell CLL/lymphoma 6	6.06	8.66	6.07	8.02	3.88	1.56
CSF2	Colony stimulating factor 2 (granulocyte-macrophage) (GMCSF)	7.48	10.04	5.90	9.24	3.39	1.74
PTGS2	Prostaglandin-endoperoxide synthase 2 (COX2)	8.88	11.38	5.65	10.37	2.81	2.01
CD69	CD69 molecule	9.04	11.52	5.56	10.49	2.72	2.04
HEY1	Hairy/enhancer-of-split related with YRPW motif 1	9.18	11.62	5.43	10.50	2.49	2.18
EGR1	Early growth response 1	8.08	10.41	5.05	10.02	3.85	1.31
TRIB1	Tribbles homolog 1	7.37	9.70	5.02	8.47	2.14	2.35
GBP2	Guanylate binding protein 2	6.87	9.01	4.40	8.26	2.62	1.68
IER3	Immediate early response 3	10.66	12.78	4.34	12.31	3.13	1.39
LRRC8B	Leucine rich repeat containing 8 family, member B	8.56	10.66	4.30	9.69	2.20	1.96
ANKRD57	Ankyrin repeat domain 57	8.51	10.57	4.17	9.32	1.75	2.39
TARP	Ti antigen CD3-associated protein gamma chain V-J-C region precursor	4.13	6.19	4.17	4.82	1.62	2.58
RAB31	member RAS oncogene family	7.42	9.47	4.14	8.19	1.71	2.42
CCL3L1	Chemokine ligand 3-like 1	9.63	11.56	3.82	11.14	2.86	1.33
PTGER4	Prostaglandin E receptor 4	9.63	11.53	3.73	10.81	2.26	1.65
CCL2	Chemokine ligand 2	9.84	11.71	3.66	10.50	1.58	2.31

LTE_4 and LTD_4 stimulated LAD2 cells were compared to vehicle control as well as LTE_4 compared to LTD_4 responses. Highlighted entries show genes of interest. Relative Intensity (RI)(log2 scale).

Hierarchical clustering of significantly (ANOVA $p<0.05$, >2 fold change) regulated genes is presented as a heatmap (analysed using Partek Genomics Suite).

4.2 Study Aims

LTE_4 induces potent responses in LAD2 cells offering an interesting model to identify the elusive GPCR responsible for LTE_4 signalling. LAD2 cells have already been shown to proliferate and release mediators upon LTE_4 stimulation equal to or greater than LTD_4 (170,254,257). Our own microarray analysis of LAD2 cells has also confirmed LTE_4's potency supporting published data and identified new leukotriene-regulated genes, such as GMCSF. LUVA cells are another mast cell line that was spontaneously generated from peripheral blood $CD34^+$ cell cultures and have morphology similar to that of LAD2 cells i.e. they contain tryptase and chymase, express c-Kit and IgE receptors and degranulate on cross-linking of FcεRI (219). qRT-PCR analysis has shown detectable levels of CYSLTR1 and CYSLTR2 but their responses to cysteinyl leukotriene stimulation are as yet to be characterised (219).

The aim of this study is to identify GPCR receptor(s) responsible for LTE_4 signalling in human mast cells. Cysteinyl leukotriene-induced signalling and gene expression will be compared between two human mast cell lines, LAD2 and LUVA, in order to characterise a receptor responding to LTE_4.

4.3 Results

4.3.1 Analysis of cysteinyl leukotriene-induced responses in human mast cells

In our initial microarray studies, LTD_4 and LTE_4 were shown to robustly regulate gene expression in the human mast cell line, LAD2. To analyse this further, the relative responsiveness to the cysteinyl leukotrienes, LTD_4 and LTE_4, specifically looking at genes that were highly upregulated in the microarray study i.e. PTGS2 (COX2), CCL4 and GMCSF, was analysed in two human mast cell lines, LAD2 and LUVA. qRT-PCR analysis of LAD2 cells stimulated with cysteinyl leukotrienes for 2 hours showed induction of COX2, CCL4 and GMCSF expression with LTE_4 consistently matching or being the more potent of the two ligands (Figure 4.2A). LUVA cells stimulated with LTD_4 induced gene expression in a similarly robust way to LAD2 cells, however LTE_4 induced only very weak responses (Figure 4.2B).

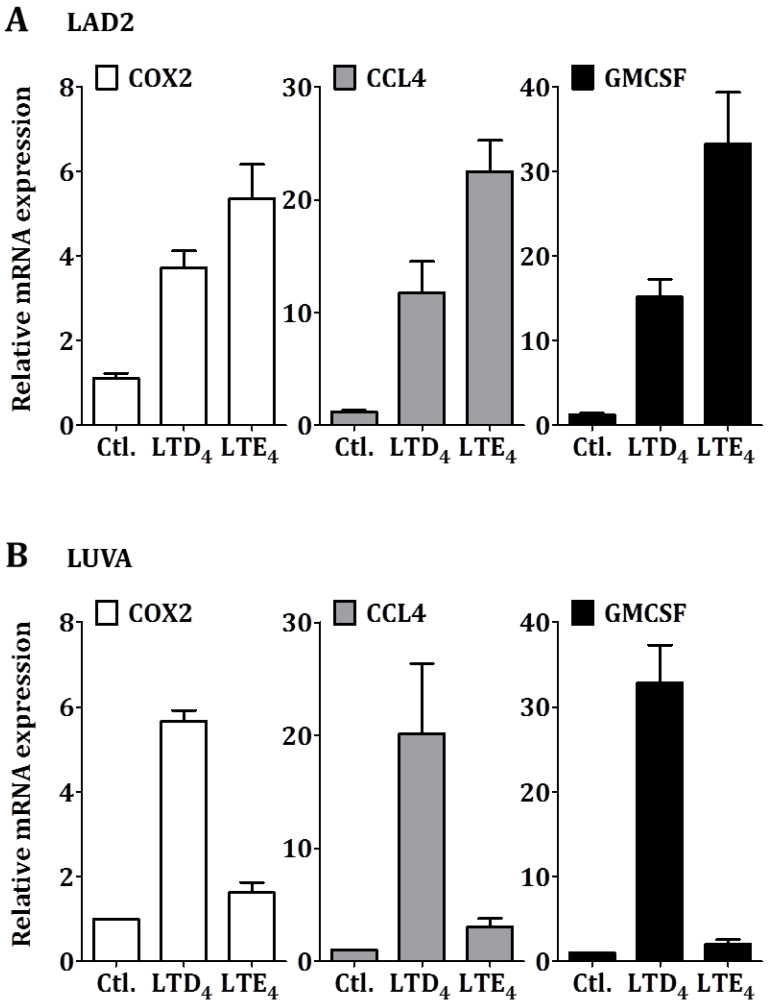


Figure 4.2 Human mast cell lines respond to cysteinyl leukotriene stimulation.
Human mast cell responsiveness was analysed to cysteinyl leukotriene stimulation in the presence of L-cysteine 3 mmol/L. Relative quantification of PTGS2 (COX2), CCL4 and GMSCF mRNA in human mast cell lines **(A)** LAD2 and **(B)** LUVA when stimulated for 2 hours with either vehicle control, LTD_4 100 nmol/L or LTE_4 100 nmol/L, data compared to vehicle control, 18s used as an endogenous control, *n*=3. All data expressed as mean ± SEM.

As intracellular calcium mobilisation is a secondary messenger signalling cue for $CysLT_1$ and $CysLT_2$ (Figure 3.2C-D), cysteinyl leukotrienes induced calcium mobilisation was analysed in our two human mast cell lines. In LAD2 cells, all cysteinyl leukotrienes induced a concentration-dependent flux in intracellular calcium (Figure 4.3A). LTD_4 and LTC_4 were very similar in potency and LTE_4 was the weakest of all three ligands but still induced a robust response. In contrast, in LUVA cells, LTD_4 was the most potent ligand followed by LTC_4, while LTE_4 induced very weak, non-specific fluxes in intracellular calcium (Figure

4.3B). The order of ligand potencies for inducing intracellular calcium mobilisation in LUVA cells was very similar to that of $CysLT_1$ transfected HEK293 cells (Figure 3.2C).

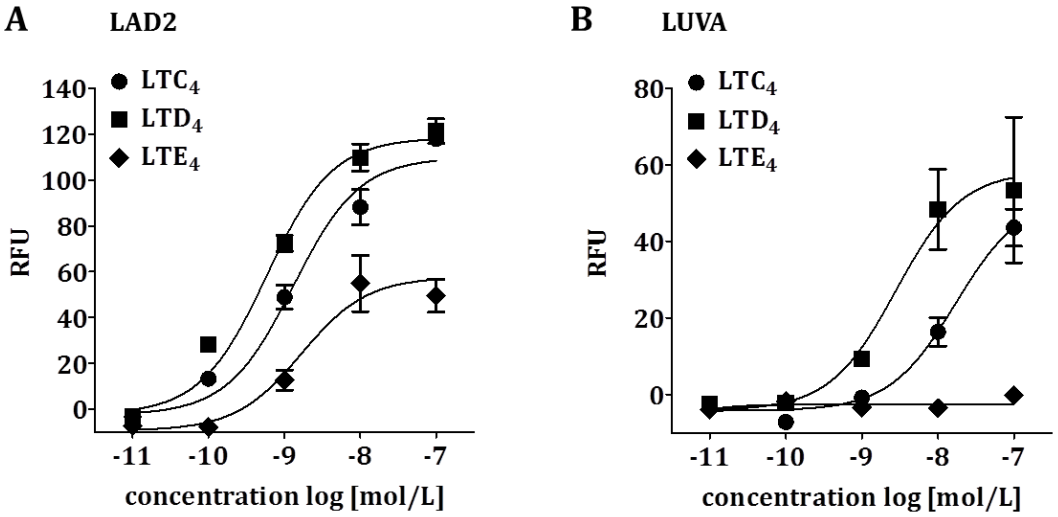


Figure 4.3 Cysteinyl leukotrienes induce intracellular calcium mobilisation in human mast cell lines.

Human mast cell lines were analysed for intracellular calcium mobilisation upon stimulation. Baseline corrected peak intracellular calcium fluxes of **(A)** LAD2 and **(B)** LUVA cells stimulated with the indicated concentrations of LTC_4 (circle), LTD_4 (square) and LTE_4 (diamond). Data from 2-3 experiments run in triplicate, presented as mean ± SEM. Relative fluorescence units (RFU).

4.3.2 Comparison of GPCR gene expression profiles between LAD2 and LUVA cells

LAD2 and LUVA cells represent two human mast cell lines that respond differently to LTE_4 stimulation. Previous studies have suggested that LTE_4 signals in LAD2 cells through a different receptor than $CysLT_1$ so it could be hypothesised that the lack of LTE_4 signalling in LUVA cells could be due to a lack of expression (or lower expression) of the elusive, LTE_4 preferential receptor (257). Using our Affymetrix Human Gene 1.0 ST microarray data, gene transcript relative intensities were compared in LAD2 and LUVA cells using Partek Genomics Suite and a list of genes expressed differently between cell lines (ANOVA $p<0.05$; 2 fold difference) was generated. GPCR genes were filtered using GPCR databases from IUPHAR (119). 27 GPCRs differed significantly in LAD2 cells with a change in relative intensities of more than 2-fold (Figure 4.4, Table 4.1). Analysing all 27 differentially expressed GPCR would be inappropriate as many (17 GPCRs) are already well characterised GPCRs, for example the adrenergic β_2 receptor (ADRB2), the histamine H4

receptor (HRH4) and the chemokine receptors, CCR4 and CXCR3. Target GPCRs were identified based on expression levels from the group of 10 GPCRs that were considered orphans; GPR12, GPR37, GPR65, GPR85, GPR114, GPR137B, GPR174, MAS1L, MRGPRX2 and P2RY8. GPR65, MAS1L and MRGPRX2 (shown in grey, Figure 4.4) were the most differentially expressed orphan GPCRs (9.9, 32.4 and 70.2 fold change LAD2 vs. LUVA respectively) and so were considered the most promising targets for further study. Interestingly, $CysLT_1$ was also a GPCR that was identified as being differentially expressed in LAD2 cells compared to LUVA (shown as open bar, Figure 4.4, 4.3 fold change).

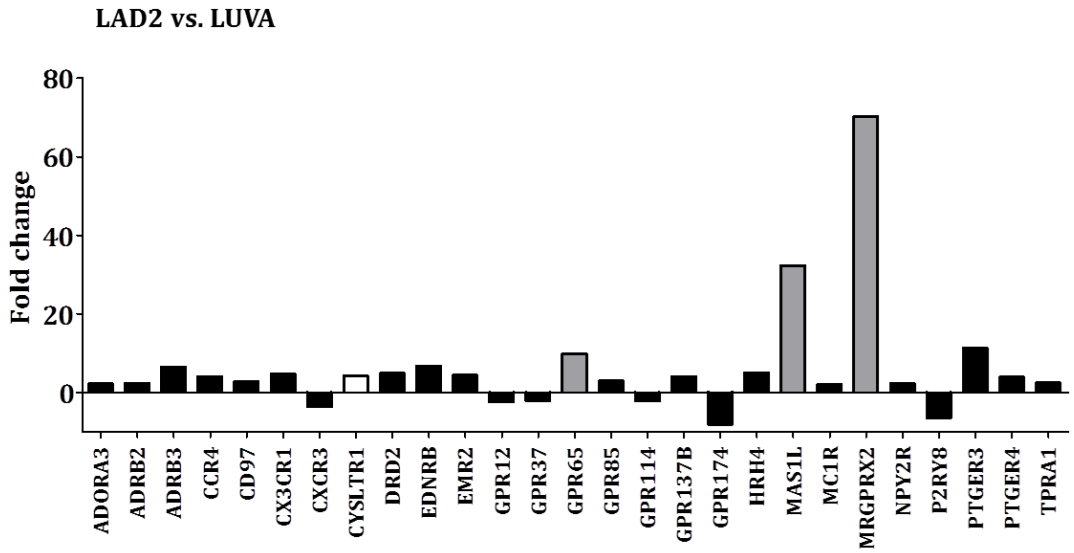


Figure 4.4 Comparison of differential GPCR expression between LAD2 and LUVA cells.

Analysis of differential GPCR mRNA expression levels in unstimulated LAD2 and LUVA cells. Fold change of relative intensities of mRNA expression levels of LAD2 cells compared to LUVA cells. Data represented as comparisons > 2 fold and $p<0.05$ ANOVA, grey bars represent genes of interest and open bar denotes CYSLTR1.

Table 4.2 Differentially expressed GPCRs in LAD2 cells compared to LUVA cells (more than 2 fold difference).

Gene	Description	Orphan?	Fold Change LAD2 vs. LUVA
ADORA3	Adenosine A3 receptor	No	2.27
ADRB2	Adrenergic β2 receptor	No	2.39
ADRB3	Adrenergic β3 receptor	No	6.52
CCR4	Chemokine receptor	No	4.16
CD97	Adhesion class receptor	No	2.87
CX3CR1	Chemokine receptor	No	4.76
CXCR3	Chemokine receptor	No	-3.60
CYSLTR1	Cysteinyl leukotriene receptor 1	No	4.32
DRD2	Dopamine receptor D2	No	4.90
EDNRB	Endothelin receptor type B	No	6.83
EMR2	Adhesion class receptor	No	4.50
GPR12		Yes	-2.41
GPR37		Yes	-2.05
GPR65		Yes	9.93
GPR85		Yes	2.94
GPR114	Adhesion class receptor?	Yes	-2.14
GPR137B		Yes	4.12
GPR174		Yes	-8.04
HRH4	Histamine H4 receptor	No	5.06
MAS1L		Yes	32.41
MC1R	Melanocortin 1 receptor	No	2.02
MRGPRX2		Yes	70.23
NPY2R	Neuropeptide Y2 receptor	No	2.30
P2RY8		Yes	-6.34
PTGER3	Prostaglandin E receptor 3	No	11.40
PTGER4	Prostaglandin E receptor 4	No	3.96
TPRA1	Transmembrane protein, adipocyte associated 1	No	2.52

To ascertain whether LTE_4 could mediate signal transduction through either of the target genes identified, GPR65, MAS1L or MRGPRX2, models of HEK293T cells overexpressing genes of interest were generated with or without the co-expression of h-$G\alpha_{16}$. Intracellular calcium mobilisation was analysed within these transfectants upon stimulation with cysteinyl leukotrienes. [$CysLT_1$] transfectants were run in parallel as a control for the experiments. LTC_4 and LTD_4 induced intracellular calcium mobilisation in [$CysLT_1$] transfectants with very weak responses upon LTE_4 stimulation (Figure 4.5A-F). Unfortunately, cysteinyl leukotriene stimulation did not induce any specific fluxes in intracellular calcium in any of our target transfectants (Figure 4.5A-F).

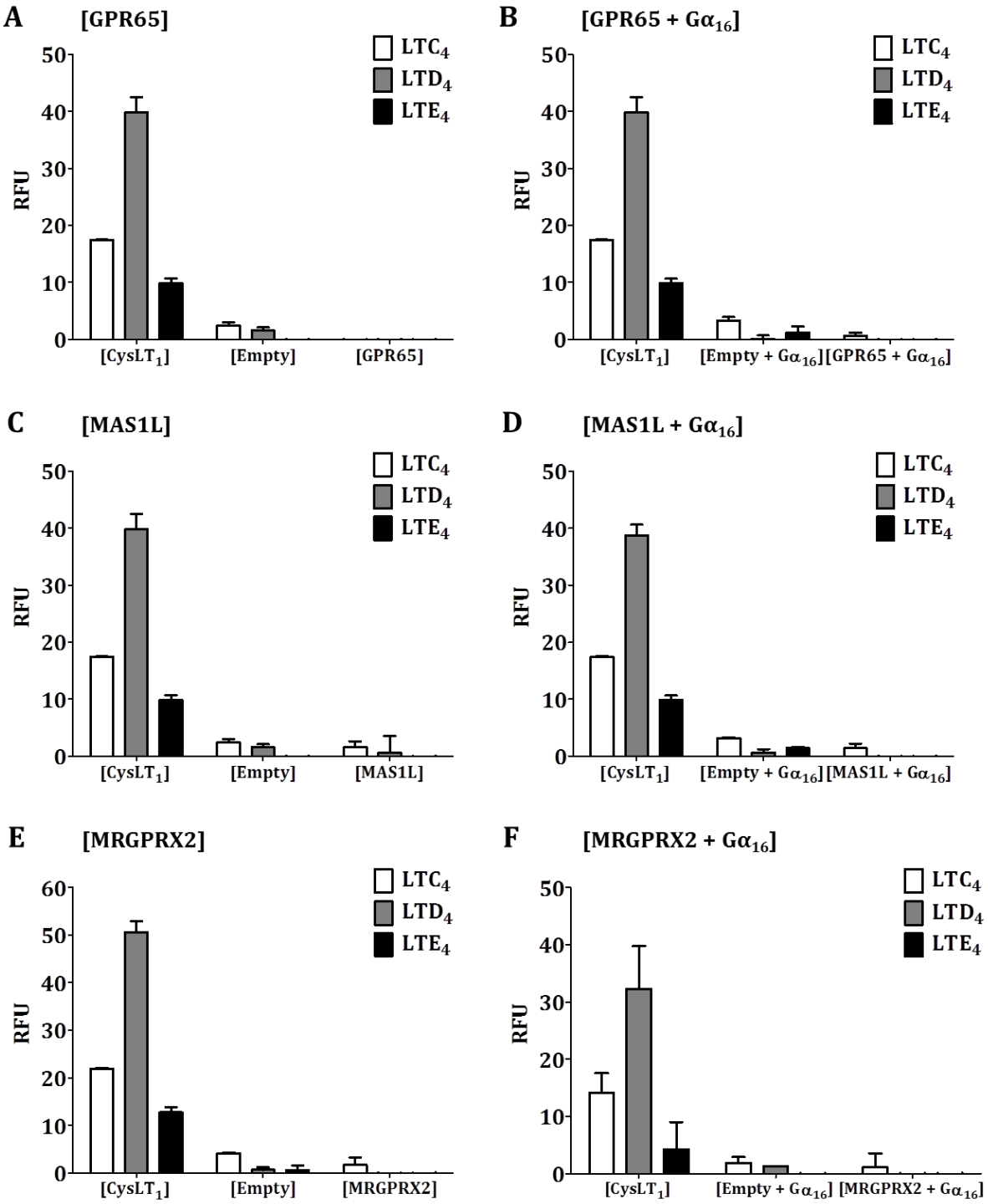


Figure 4.5 Cysteinyl leukotrienes do not activate GPR65, MAS1L or MRGPRX2 receptors.

Intracellular calcium mobilisation was analysed in models of HEK293T cells transiently transfected with the genes of interest. Baseline corrected peak intracellular fluxes of **(A)** [GPR65] **(B)** [GPR65 + $G\alpha_{16}$] **(C)** [MAS1L] **(D)** [MAS1L + $G\alpha_{16}$] **(E)** [MRGPRX2] **(F)** [MRGPRX2 + $G\alpha_{16}$] transfectants stimulated with either LTC_4 100 nmol/L, LTD_4 100 nmol/L or LTE_4 100 nmol/L, all with control [$CysLT_1$] and the appropriate empty vector

control. Data from 2-3 experiments run in triplicate, presented as mean ± SEM. Relative fluorescence units (RFU).

4.3.3 Analysis of cysteinyl leukotriene responses using selective receptor inhibitors

To determine whether $CysLT_1$ could be involved in LTE_4 signal transduction, LAD2 and LUVA cells were pretreated with selective $CysLT_1$ and $CysLT_2$ receptor antagonists, Montelukast and HAMI 3379, respectively. Antagonist selectivity was previously verified in HEK293T cell transfection models (Figure 3.2E-F). qRT-PCR analysis of COX2 and CCL4 in LAD2 cells showed that both LTD_4 and LTE_4 signalling was sensitive to Montelukast while HAMI 3379 had no affect (Figure 4.6A). In LUVA cells, LTD_4 signalling was again sensitive to Montelukast pretreatment but not to HAMI 3379 (Figure 4.6B). Responses to LTE_4 in LUVA cells, as previously described (Figure 4.2B), were very weak so it is difficult to draw a conclusion as to whether its response was susceptible to either antagonist. Analysis of intracellular calcium mobilisation in these cells showed a very similar picture. LTD_4-induced calcium mobilisation was sensitive to Montelukast pretreatment in both LAD2 and LUVA cells while HAMI 3379 had no affect (Figure 4.6C-D). LTE_4-induced calcium responses were inhibited only by Montelukast in LAD2 cells. Again the very weak calcium responses to LTE_4 in LUVA did not allow for any proper analysis of selective inhibition (Figure 4.6D).

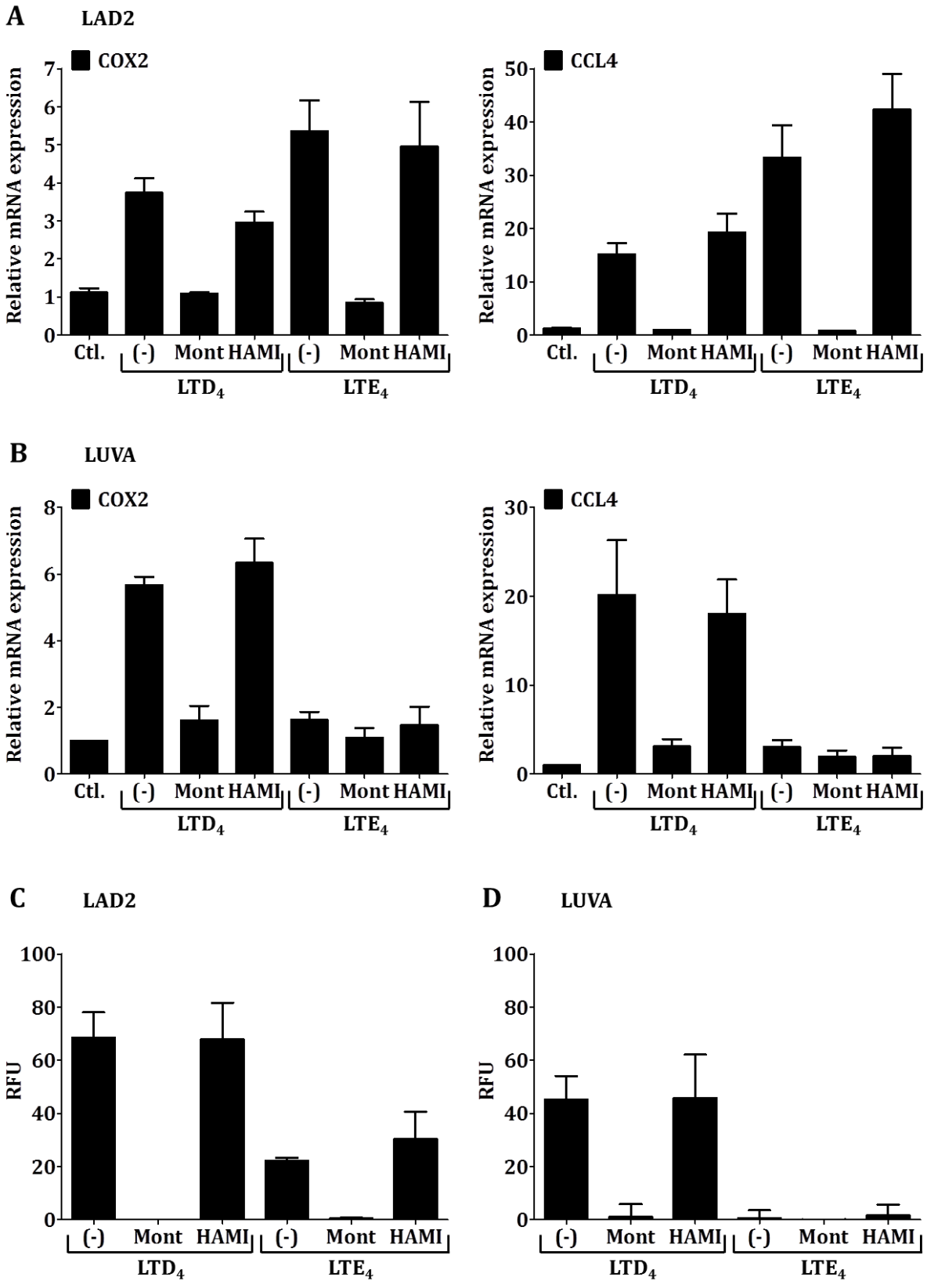


Figure 4.6 Cysteinyl leukotriene responses are sensitive to $CysLT_1$ antagonism in human mast cell lines.

Human mast cell lines were analysed for their sensitivity to $CysLT_1$ or $CysLT_2$ antagonism upon cysteinyl leukotriene stimulation. Relative quantification of COX2 and CCL4 mRNA in

(A) LAD2 and **(B)** LUVA cells stimulated for 2 hours with either vehicle control, LTD_4 100 nmol/L or LTE_4 100 nmol/L with or without a 10 minute pre-incubation with either "Mont" (Montelukast) 100 nmol/L or "HAMI" (HAMI 3379) 1 µmol/L, all in the presence of L-cysteine 3 mmol/L, data compared to vehicle control, 18s used as endogenous control, *n*=3. Baseline corrected peak calcium flux data of human mast cell lines **(C)** LAD2 and **(D)** LUVA stimulated with either vehicle control, LTD_4 100 nmol/L or LTE_4 100 nmol/L with or without a 15 minute pre-incubation with Montelukast 100 nmol/L or HAMI 3379 1 µmol/L, *n*=3. All data represented as mean ± SEM. Relative fluorescence units (RFU).

4.3.4 Analysis of cysteinyl leukotriene receptor knockdown in LAD2 cells

To verify whether the potent LTE_4-induced, Montelukast sensitive, response in LAD2 cells was attributable specifically to $CysLT_1$ signalling and not via another Montelukast sensitive receptor, $CysLT_1$ and $CysLT_2$ receptor knockdowns were generated in LAD2 cells. Four shRNA targeting different regions of CYSLTR1 or CYSLTR2 were transduced into separate LAD2 cell populations using lentiviral particles (see section 2.5 *Preparation of lentiviral particles and transduction of human mast cells* for shRNA specifications). qRT-PCR analysis of $CysLT_1$ transfectants revealed shRNA "475" successfully knocked down CYSLTR1 without significantly affecting CYSLTR2 mRNA expression (Figure 4.7A). While in the $CysLT_2$ transfectants, shRNA "190" and "324" significantly knocked down CYSLTR2 mRNA while not significantly affecting CYSLTR1 (Figure 4.7B). As shRNA "190" was able to knockdown CYSLTR2 mRNA to a greater extent than "324", this as well as LAD2 populations transduced with shRNA "475" were continued for analysis.

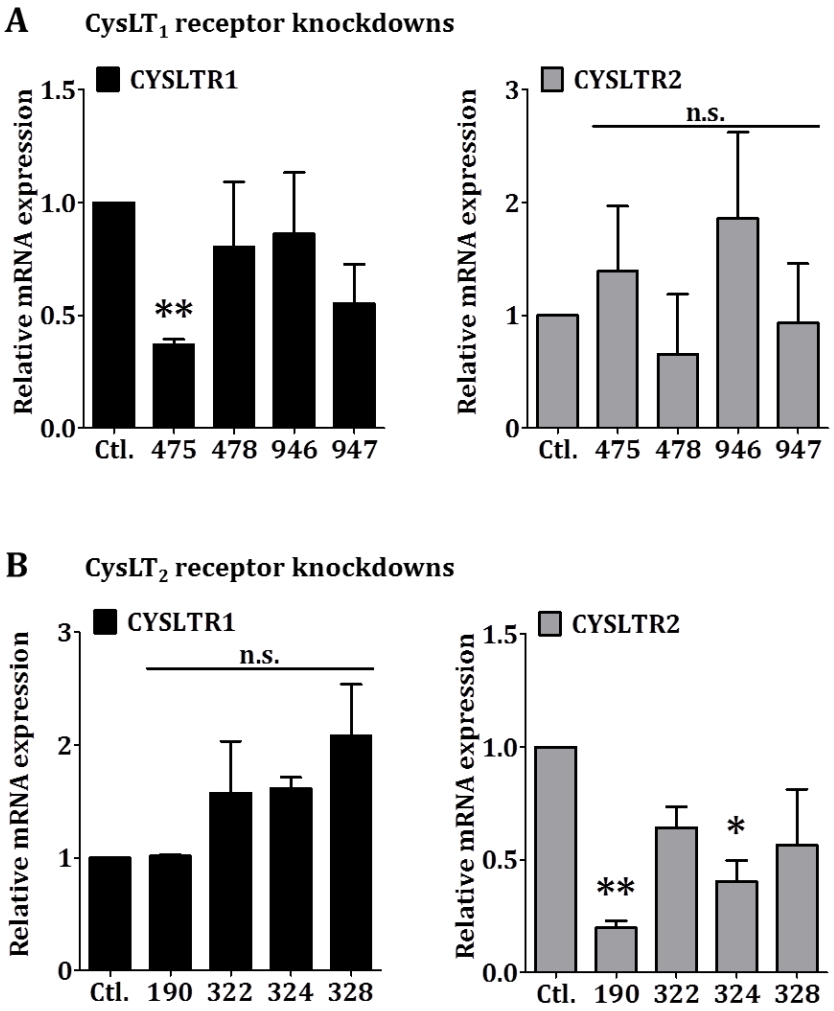


Figure 4.7 Validation of $CysLT_1$ and $CysLT_2$ receptor knockdown in LAD2 cells.
LAD2 cells stably transfected with the indicated CYSLTR1 and CYSLTR2 shRNA were analysed by qRT-PCR for successful gene knockdown. Relative quantification of CYSLTR1 and CYSLTR2 mRNA in LAD2 cells transfected with shRNA targeting different regions of **(A)** $CysLT_1$ and **(B)** $CysLT_2$ receptor coding regions. *n*=3, mean ± SEM.

To ascertain whether knocking down of either of $CysLT_1$ or $CysLT_2$ had an effect on cysteinyl leukotriene-induced responses, intracellular calcium mobilisation was analysed in $CysLT_1$ and $CysLT_2$ knockdown LAD2 cells. Knocking down of $CysLT_1$ substantially inhibited fluxes in intracellular calcium mobilisation in response to cysteinyl leukotriene stimulation (Figure 4.8A-C). At their optimal concentrations (10 nmol/L), LTC_4, LTD_4 and LTE_4-induced responses were inhibited by 69.4, 55.9 and 63.1 % (respectively, mean), when compared to control cells. No such inhibition was observed in $CysLT_2$ knockdown LAD2 cells with LTC_4, LTD_4 and LTE_4 dose-dependent curves matching that of the control cells (Figure 4.8D-F). qRT-PCR analysis of $CysLT_1$ and $CysLT_2$ knockdowns in LAD2 showed a similar outcome (Figure 4.9). COX2 CCL4 and GMCSF mRNA expression upon

LTD_4 and LTE_4 stimulation were almost completely abrogated in $CysLT_1$ knockdown LAD2 cells while knocking down of $CysLT_2$ had no real effect.

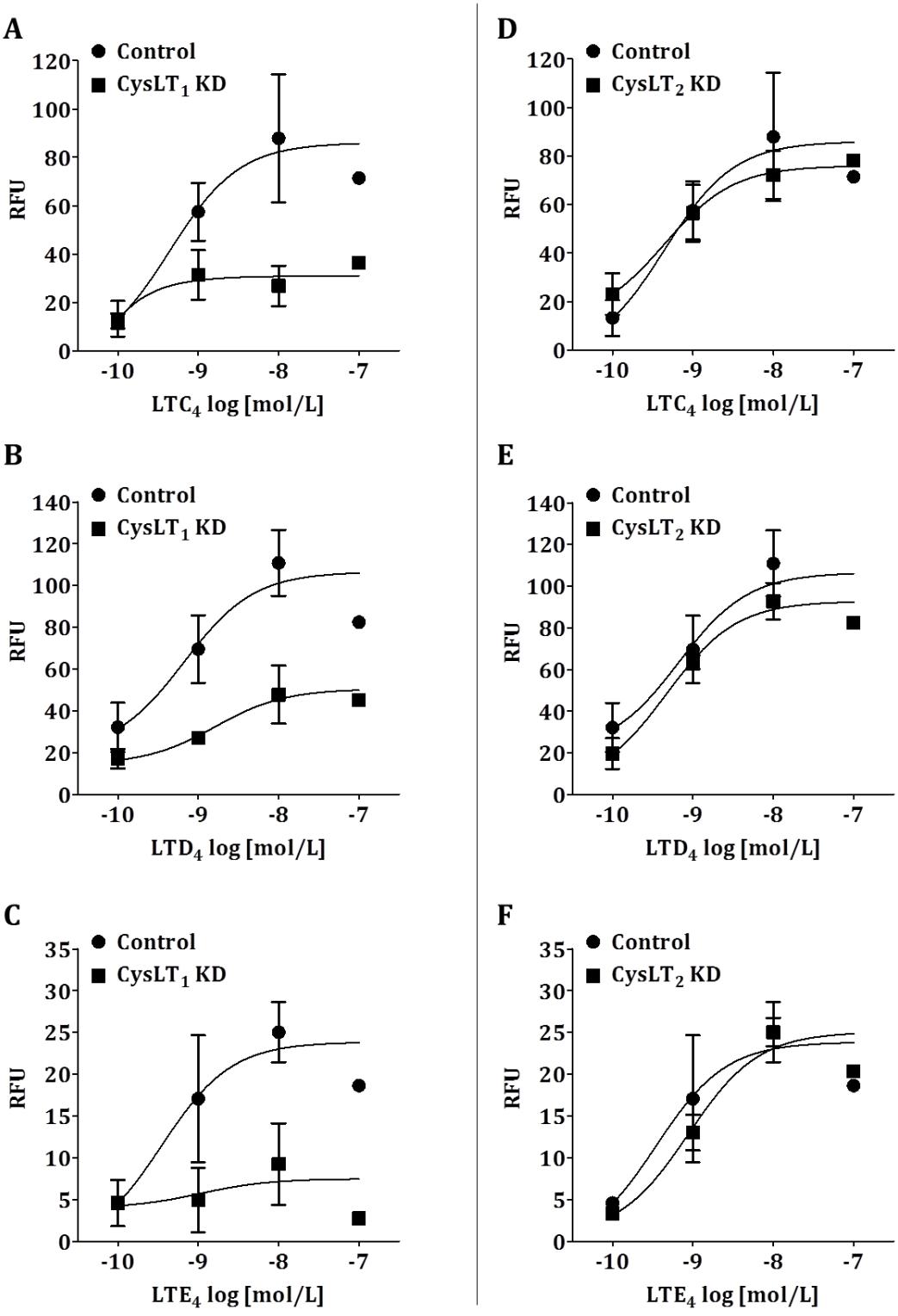


Figure 4.8 $CysLT_1$ knockdown in LAD2 cells significantly reduces intracellular calcium mobilisation to cysteinyl leukotriene stimulation.

Baseline corrected peak calcium flux data of LAD2 cells stably transduced with lentivirus containing shRNA targeting **(A-C)** CYSLTR1 and **(D-F)** CYSLTR2 with empty vector

transduced cells used as control. Cells were stimulated with the indicated concentrations of either LTC_4, LTD_4 or LTE_4. Data from 1-3 experiments, run in triplicate, presented as mean ± SEM. Knockdown (KD). Relative fluorescence units (RFU).

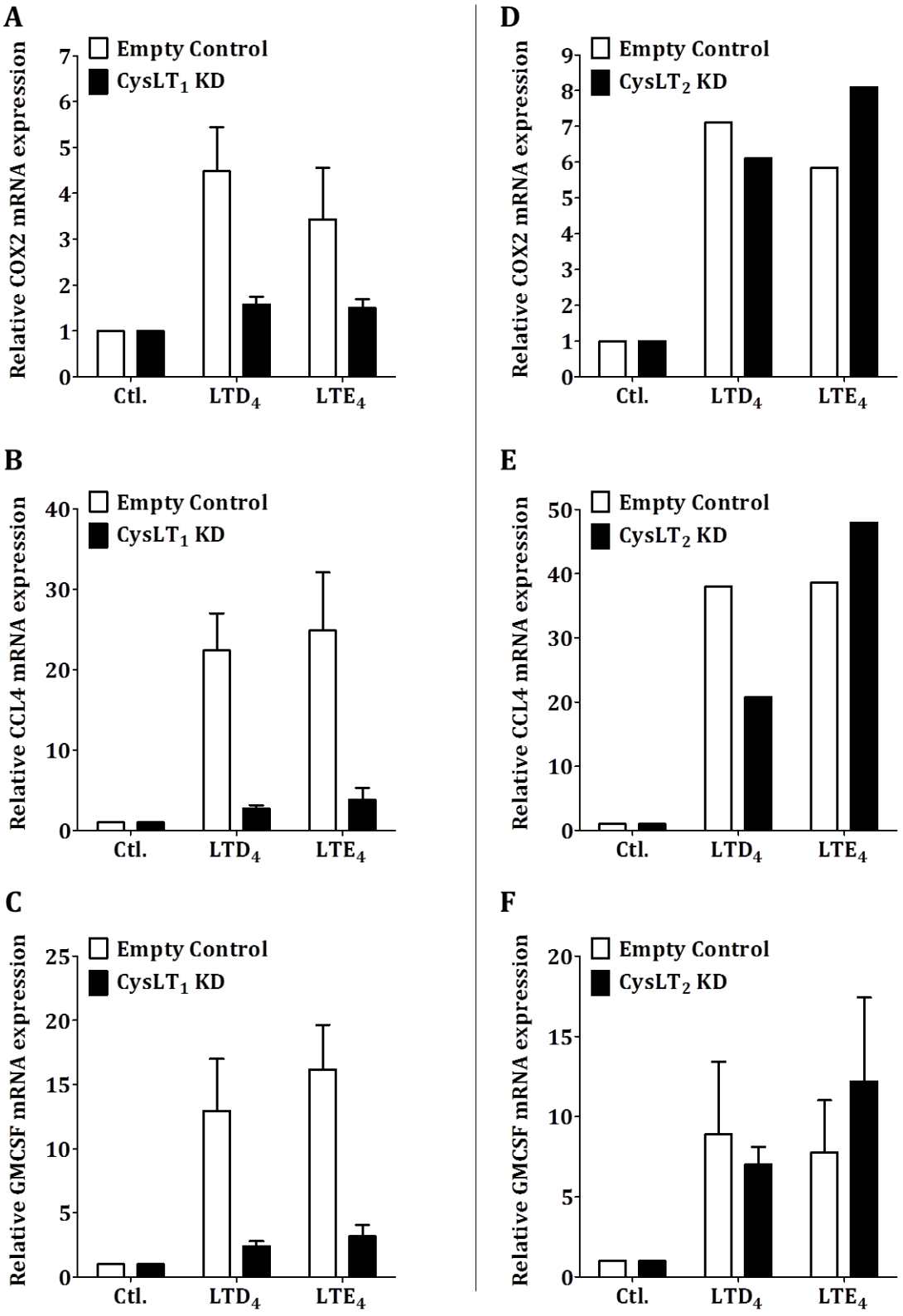


Figure 4.9 $CysLT_1$ knockdown in LAD2 cells significantly reduces gene up-regulation in response to cysteinyl leukotriene stimulation.

Relative quantification of COX2, CCL4 and GMCSF mRNA expression of LAD2 cells stably transfected with lentivirus containing shRNA targeting **(A-C)** CYSLTR1 and **(D-F)** CYSLTR2 with empty vector transduced cells used as control. Cells were stimulated with

either LTD_4 100 nmol/L or LTE_4 100 nmol/L for 2 hours in the presence of L-cysteine 3 mmol/L, data relative to vehicle control ("Ctl."), 18s used as endogenous control, **(A-C,F)** *n*=3-5, **(D-E)** *n*=1. All data expressed as mean ± SEM. Knockdown (KD).

4.4 Discussion

Mast cells are one of only a few cell types identified that potently respond to LTE_4 stimulation (254,257). Due to the rarity of primary tissue-derived human mast cells, lines spontaneously derived from $CD34^+$ cell cultures have dominated the field of human mast cell research. LAD2 cells are considered the best studied human mast cell line, deemed to be more representative of tissue-derived human mast cells than any other line previously generated (19,256). Paruchuri *et al.* have already shown that they effectively respond to cysteinyl leukotriene stimulation and it was considered the best cell line to study in order to identify the LTE_4 preferential receptor (257). LUVA cells have been recently generated and their responses to cysteinyl leukotriene stimulation have not been previously analysed (219). So using LAD2 and LUVA cells as a comparison, this study was undertaken to identify the GPCR responsible for LTE_4 preferential signalling.

Microarray data of LAD2 cells, generated in our laboratory, revealed that LTE_4 is more potent at inducing gene expression than LTD_4. This observation was confirmed by qRT-PCR at an mRNA level for specific genes, such as COX2, CCL4 and GMCSF (Figure 4.2A). Surprisingly another mast cell line, LUVA, showed very weak responses to LTE_4 even though LTD_4-induced mRNA expression was at a similar level as in LAD2 cells (Figure 4.2B). This discrepancy between the two mast cell lines was further extended when comparing cysteinyl leukotriene-induced intracellular calcium mobilisation (Figure 4.3). Even though LTE_4 induced the weakest responses in LAD2, the dose-dependent intracellular fluxes were very robust. The response to LTE_4 in LUVA cells was negligible, with non-specific fluxes in intracellular calcium. This lack of response to LTE_4 and the pattern of cysteinyl leukotriene potencies in LUVA cells mimic the initial observations of "classical" $CysLT_1$ signalling seen in literature and in our over-expression models (Figure 3.2)(114,239).

This lack of LTE_4 responsiveness in LUVA cells led us to postulate that an additional GPCR, the LTE_4 preferential GPCR, was functionally expressed in LAD2 cells and not in LUVA. Comparison of microarray data from both cell lines revealed 10 differentially expressed orphan GPCRs. 3 of these orphan GPCRs were significantly overexpressed in LAD2 cells compared to LUVA but unfortunately when tested none of these target GPCRs responded to cysteinyl leukotriene stimulation (Figure 4.5). Since the study was conducted, GPR65

has been suggested as a proton-sensing GPCR involved in the regulation of matrix metalloproteinases (258,259). MRGPRX2 has been suggested to signal via β-defensins while MAS1L remains relatively unstudied (260,261). However all three are still considered class A orphan GPCRs according to the IUPHAR databases (119,258-261).

Interestingly, $CysLT_1$ was also differentially expressed with a 4.32 fold increase in LAD2 cells compared to LUVA. Responses to LTD_4 and LTE_4 in LAD2 cells (as well as LUVA) were sensitive to the $CysLT_1$ antagonist, Montelukast (Figure 4.6). Cysteinyl leukotriene sensitivity to $CysLT_1$ antagonism in human mast cells has been well documented in the literature but with another selective $CysLT_1$ antagonist, MK-571(170,254,257). As previously described, both Montelukast and MK-571 were synthesised before any cysteinyl leukotriene receptor had been cloned and characterised (106,109,262). Numerous studies have indicated that both antagonists may have anti-inflammatory actions that are $CysLT_1$-independent, suggesting that they also act via other GPCRs with similar sequence homology (263-267). Knockdown of $CysLT_1$ in LAD2 cells elucidated that the signalling pathway of LTE_4 within this cell system is $CysLT_1$-dependent. Both the mobilisation of intracellular calcium and upregulation of mRNA expression of COX2, CCL4 and GMCSF to cysteinyl leukotriene stimulation were attenuated in these $CysLT_1$ knockdowns while knocking down of $CysLT_2$ had no effect (Figure 4.8 and Figure 4.9).

This LTE_4-$CysLT_1$-dependent pathway in LAD2 cells is contradictory to previous observations. Paruchuri *et al.* has analysed $CysLT_1$ knockdown LAD2 cells in two separate studies (170,257). Although LTD_4 responses were consistently attenuated by $CysLT_1$ knockdown, the responses to LTE_4 were only slightly inhibited when analysing CCL4 release and had no effect on PGD_2 release. Lentiviral transfection of shRNA were both used in Paruchuri *et al.*'s studies and our study to knockdown $CysLT_1$, although slight differences in methodologies and analysis targets might account for the differences seen. Firstly, different regions of the CYSLTR1 gene were targeted which might affect how significantly these responses to LTE_4 are altered. Analysing several different shRNA's targeting different regions of the gene of interest is important for acquiring the optimal efficiency of knockdown (Figure 4.7). Secondly, our transfectants underwent puromycin selection unlike in the Paruchuri *et al.* studies, a process used to obtain a stable population of LAD2 cells of which over 95 % were expressing GFP (the shRNA reporter gene). This allowed the cells to recover from the transfection procedure and more importantly increased the proportion of cells that had incorporated the shRNA which might increase the ability to discriminate whether a knockdown has had an effect. Finally, different outputs were analysed. Intracellular calcium mobilisation is our most sensitive tool for analysing initial GPCR signalling events, with responses seen within seconds of

stimulation. These responses are unaffected by potential secondary auto/paracrine signalling from other mediators released upon cysteinyl leukotriene stimulation that might potentially be seen with longer stimulations to analyse protein/lipid release.

This study has shown that LTE_4-induced signalling response in LAD2 cells involves the classical cysteinyl leukotriene receptor, $CysLT_1$.

Chapter 5

Comparison of CYSLTR1 genetic variability between LAD2 and LUVA cell lines

5.1 Introduction

Polymorphisms within DNA are a source of diversity that affects phenotypes ranging from hair colour to disease susceptibility. The most common are single nucleotide polymorphisms (SNPs) which can alter single base pairs and can occur in both coding and non-coding regions of genes. SNPs that affect the coding region are categorised as either nonsynonymous or synonymous i.e. they do or do not affect the protein sequence. Nonsynonymous SNPs can lead to either the resulting codon encoding a different amino acid affecting protein structure or even to a non-functional protein (missense) or the resulting codon being a termination codon leading to truncated proteins (nonsense). SNPs that occur within the non-coding region are more complex as they have no effect on actual protein sequence. It is thought that they could alter processes such as transcript expression, splice variation, transcription factor binding and mRNA degradation that could in turn significantly affect downstream cell functions.

As previously stated, the CYSLTR1 gene resides on chromosome Xq13.2-q21.1 and encodes a protein that is 337 amino acids long. The major transcript, which has been identified in many cell types, contains three exons in which the third exon contains the full coding region and is intronless (118,268). Numerous SNPs have been identified in the promoter and coding region of CYSLTR1, shown in Figure 5.1 (further information in Table 5.1). In the promoter region, which is thought to be located between -125 and -786, four SNPs have been identified; rs321029 T>C (SNP 1), rs2637204 C>A (SNP 2), rs2806489 A>G (SNP 3) and rs7066737 C>T (SNP 4) (118).

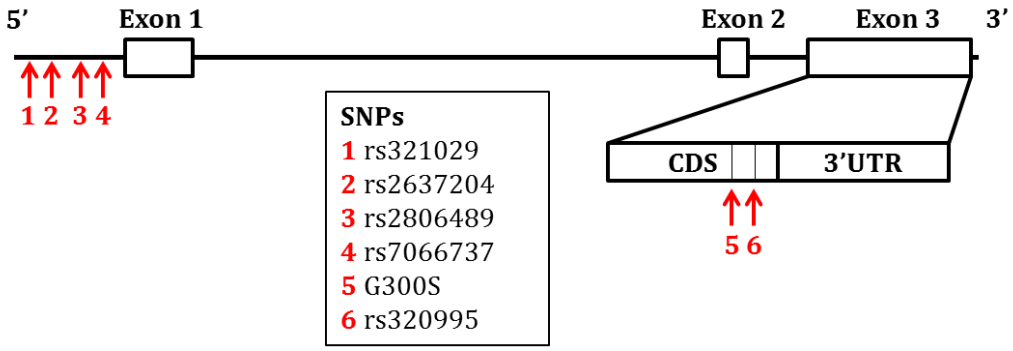


Figure 5.1 Schematic representation of the CYSLTR1 gene structure.
SNP locations are shown by red arrows, exons are shown by boxes, coding region (CDS) and 3' untranslated region (3'UTR).

Table 5.1 Single nucleotide polymorphisms in the CYSLTR1 gene.

SNP	Reference	Location	Position	Allele (reverse)	Residue Change
1	rs321029	Promoter	4342	T>C	NA
2	rs2637204	Promoter	4501	C>A	NA
3	rs2806489	Promoter	4640	A>G	NA
4	rs7066737	Promoter	4721	C>T	NA
5	G300S	Coding region	59743	G>A	G (Gly) > E (Glu)
6	rs320995	Coding region	59771	T>C	F (Phe) > F (Phe)

Position relative to CYSLTR1 gene NG_012809.1 sequence, non-applicable (NA), Glycine (Gly), Glutamic acid (Glu), Phenylalanine (Phe).

Several studies have analysed whether there are any disease associations with SNPs in the promoter region. Duroudier *et al.* found that in a British 1958 birth cohort, SNP 3 had no association with age of asthma onset, wheeze or serum total IgE levels (269). The A allele was, however, associated with increased risk of atopy in females only. SNP 4 showed no association with the above mentioned variables (269). Kim *et al.* analysed SNPs 1, 2 and 3 collectively to see whether a specific promoter haplotype had any disease associations. It was revealed that males had a higher risk of aspirin-sensitive asthma with the CYSLTR1 promoter haplotype TCG (SNPs 1-2-3) and a lower risk of disease with haplotype CAA (270). There were no associations made in females. This TCG haplotype was shown to have higher promoter activity than CAA which could relate to higher $CysLT_1$ expression (270). This increased TCG promoter activity was further confirmed by Zhang *et al*. although in their study of a Japanese cohort, no associations were made with development of asthma or rhinitis with any promoter haplotype (SNPs 1-2-3) (268).

SNPs have also been shown to be located in the coding region. In a Spanish cohort of children with asthma and atopic dermatitis, Arriba-Mendez *et al.* showed that in males only there was an increased risk of allergic asthma with atopic dermatitis with the C allele of SNP 6 (Table 5.1) (271). Hong *et al.* analysed two separate Chinese populations and were able to associate SNP 6 with increased risk of asthma but this was comparable in both males and females. However, Hao *et al.* and Zhang *et al.* found no associations with asthma related phenotypes with SNP 6, with the former study linking SNP 6 to atopy severity in females, with the T allele associated with higher severity than the C allele (272). A novel SNP identified in a Tristan da Cunha population, G300S (SNP 5), has been also associated with an increased risk of atopy and asthma (273). This $CysLT_1$ variant was shown, when transfected into HEK293 cells, to have a significant reduction in EC_{50} when analysing LTD_4-induced intracellular calcium mobilisation (273).

5.2 Study Aims

There is compelling evidence that genetic variations in the CYSLTR1 gene could lead to increased risk of asthma and allergy. This could be due to altered receptor functionality as shown for SNPs in both the promoter and the coding region (268,270,273). Our data has suggested that cysteinyl leukotrienes signal through $CysLT_1$ in both LAD2 and LUVA cells but there is differential LTE_4 signalling that is currently unexplained (see Chapter 4). LTE_4 can induce intracellular calcium mobilisation to a greater extent in LAD2 cells compared to LUVA and is just as potent at inducing CCL4, COX2 and GMCSF mRNA expression as LTD_4 in LAD2 cells. In LUVA cells, the responses to LTE_4 are weak and are similar to the initial observations made by Lynch *et al.* and Sarau *et al.* (113,114).

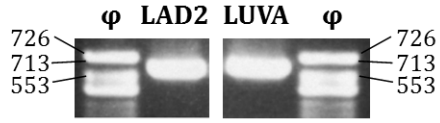
The aim of this study is to analyse whether there is any genetic variation in the CYSLTR1 gene between LAD2 and LUVA cells that could account for these observations. This will be achieved by sequencing the promoter and coding regions of CYSLTR1 in LAD2 and LUVA cells and comparing the two mast cell line sequences.

5.3 Results

5.3.1 Comparison of CYSLTR1 promoter sequence between LAD2 and LUVA cells

To determine whether there are variations in the CYSLTR1 promoter region between LAD2 and LUVA cells that may affect receptor expression and therefore functionality, DNA from both cell lines was analysed. As previously stated, the CYSLTR1 gene resides on the X chromosome. Both LAD2 and LUVA cells originate from male patients, so any allele change identified will be homozygous. PCR of DNA extracted from LAD2 and LUVA cells using primers 5'-AACTGGAGACTTGCAGGTTGCG-3' and 5'-AACATCAAAGTGCTGCCCCAGG-3' amplified a 616 base pair (bp) product (part of CYSLTR1 promoter) that was confirmed by agarose gel electrophoresis (Figure 5.2A). BLAST analysis of the sequenced PCR products from both LAD2 and LUVA cells revealed three SNPs at gene locations 4342 T>C (SNP 1), 4501 C>A (SNP 2) and 4640 G>A (SNP 3) (rs321029, rs2637204 and rs2806489 respectively, compared to CYSLTR1 RefSeqGene NG_012809.1). No point mutations were identified at any other sites including the other main promoter SNP location 4721 C=C (rs7066737) (Figure 5.2A and B-C). The sequencing data revealed that LAD2 and LUVA cells share the same promoter haplotype, "CAAC".

A



B **LAD2 promoter- haplotype CAAC**

```
4286 TAACATCAAA  GTGCTGCCCC  AGGCTTCAAT  CAGCACATAC  CAGACTGCTA  TGTCCCCTTT
4346 CTCCAGAAGC  CATATTGAGC  ACCCCACAGC  TCTAACACCT  CAGAGCCTAG  CCAAAGGCCA
4406 AGAACACTTG  CCTGATCCCC  CAAATTTCCA  ACACAGGACC  ATTGACAGCA  AGCCCAGTCA
4466 TTCCAGATCA  TCTTCAGTGG  GACAAAAAAA  GAAAAAAACA  GTTTTAATCC  AATGGAGGCA
4526 ATTTATTGTT  ATGAAGATTT  CAGGAAACAA  AGCTCAAAAA  GGAACCAGAA  AGGAAAAGGC
4586 TTTTTAGAAT  GTGGATAGAG  CCACAAGTGT  CATTAAGGAG  AGAGAGAGAG  AAACAGAGAG
4646 ATGAAATTTA  TGTTACTTAA  GATCAAGTTT  CACATAATGC  CAGTTATATT  AGCATATACT
4706 GGCAGGATTA  TCTTTCCCCA  CCTACAAATA  GTCTAATGAC  CTCACAGTCA  CAGAAATCAC
4766 AGAGAACTAA  GCTGAAGAGA  GAACACTCGT  CCCTGCTTCC  CATCTTAGAG  CAGCTGAATA
4826 ATTTCCTGAG  AATTCTATTC  CTGAAGCTAG  GAAGAAAAGT  TTATTTATAC  ATACACGCAA
4886 CCTGCAAGTC  TCCAGTTA
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C **LUVA promoter- haplotype CAAC**

```
4323 TACCAGACTG  CTATGTCCCC  TTTCTCCAGA  AGCCATATTG  AGCACCCCAC  AGCTCTAACA
4383 CCTCAGAGCC  TAGCCAAAGG  CCAAGAACAC  TTGCCTGATC  CCCCAAATTT  CCAACACAGG
4443 ACCATTGACA  GCAAGCCCAG  TCATTCCAGA  TCATCTTCAG  TGGGACAAAA  AAAGAAAAAA
4503 ACAGTTTTAA  TCCAATGGAG  GCAATTTATT  GTTATGAAGA  TTTCAGGAAA  CAAAGCTCAA
4563 AAAGGAACCA  GAAAGGAAAA  GGCTTTTTAG  AATGTGGATA  GAGCCACAAG  TGTCATTAAG
4623 GAGAGAGAGA  GAGAAACAGA  GAGATGAAAT  TTATGTTACT  TAAGATCAAG  TTTCACATAA
4683 TGCCAGTTAT  ATTAGCATAT  ACTGGCAGGA  TTATCTTTCC  CCACCTACAA  ATAGTCTAAT
4743 GACCTCACAG  TCACAGAAAT  CACAGAGAAC  TAAGCTGAAG  AGAGAACACT  CGTCCCTGCT
4806 TCCCATCTTA  GAGCAGCTGA  ATAATTTCCT  GAGAATTCTA  TTCCTGAAGC  TAGGAAGAAA
4863 AGTT
```

Figure 5.2 Comparison of the CYSLTR1 promoter haplotype in LAD2 and LUVA cells. CYSLTR1 promoter regions from genomic DNA were isolated from LAD2 and LUVA cells and analysed by DNA sequencing. **(A)** PCR products specific for amplified CYSLTR1 promoter from LAD2 and LUVA cells were run on agarose gels with the DNA ladder φX174 DNA/BsuRI (HaeIII) (φ), numbers represent size in base pairs (bp). Sequencing data of CYSLTR1 promoter region in reverse orientation from **(B)** LAD2 and **(C)** LUVA cells, red boxes represent SNP locations.

5.3.2 Comparison of CYSLTR1 coding region sequence between LAD2 and LUVA cells

To determine whether there are any variations in the protein sequence of $CysLT_1$ between LAD2 and LUVA cells that may affect receptor functionality, the coding region of CYSLTR1 from both cell lines were analysed. PCR of DNA extracted from LAD2 and LUVA cells using primers 5'- TCAATGCCTCACTACTCTTGCTTG -3' and 5'- TTGGTTTGGACTGGAAATGGG -3' amplified a 1198 bp product containing the coding region (Figure 5.3A). BLAST analysis of the sequenced PCR products revealed the presence of one point mutation at gene position 59771 C>T (SNP 6) (compared to CYSLTR1 RefSeqGene NG_012809.1) in both LAD2 and

LUVA cells (Figure 5.3B and D). SNP at this location has been recorded previously and are identified as "rs320995" (Table 5.1). This transitional mutation from cytosine to thymine was further confirmed by DNA sequencing chromatograms showing a clear peak for thymine at this SNP location (highlighted in grey, Figure 5.3C and E). The allele change from TTC to TTT is a synonymous mutation as the two codons encode the same amino acid (phenylalanine). The sequencing data revealed that the coding regions of LAD2 and LUVA cells were 100 % identical.

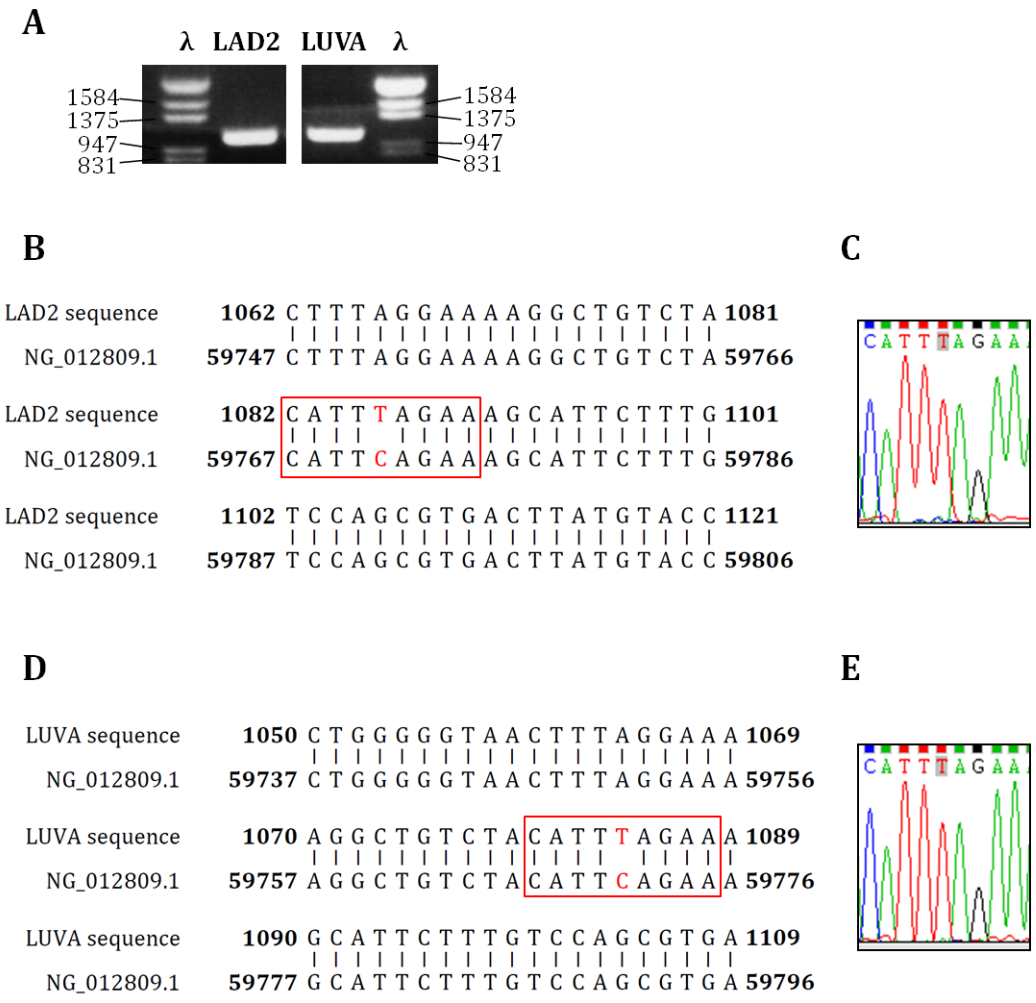

Figure 5.3 Comparison of CYSLTR1 coding regions from LAD2 and LUVA cells.
CYSLTR1 coding regions from genomic DNA isolated from LAD2 and LUVA cells were analysed. **(A)** PCR products specific for amplified CYSLTR1 coding region from LAD2 and LUVA cells were run on agarose gels with the DNA ladder λ DNA/EcoRI+HindIII (λ), numbers represent size in base pairs (bp). BLAST analysis of sequencing data of CYSLTR1 coding region in reverse orientation from **(B)** LAD2 and **(D)** LUVA cells, compared to CYSLTR1 gene NG_012809.1 sequence, data shows 60 bp extract of data with SNP location in red text, red box indicates data highlighted in DNA sequencing chromatograms **(C)** LAD2 and **(E)** LUVA, grey highlighted base represents SNP location.

5.4 Discussion

Two human mast cell lines, LAD2 and LUVA, have been shown to differentially respond to LTE_4 (see Chapter 4). On comparison of GPCR expression profiles, $CysLT_1$ was shown to be more highly expressed in LAD2 cells than LUVA and when knocked down, all cysteinyl leukotriene-induced responses were significantly reduced. This suggested that LTE_4's "full" agonist characteristics were being mediated by $CysLT_1$ but unfortunately did not explain how the responses to LTE_4 can differ significantly between the two mast cell lines. Polymorphisms such as SNPs, are the most common source of genetic variation and have been shown to affect CYSLTR1 promoter activity and responsiveness to cysteinyl leukotrienes , so it is fully possible that SNPs can alter $CysLT_1$ functionality (136,270,273). This study was undertaken to identify whether any genetic variations could account for the differential LTE_4 signalling in LAD2 and LUVA cells.

Sequencing of the promoter region identified numerous SNP sites that when compared in LAD2 and LUVA cells, were 100 % identical. They contained the promoter haplotype CAAC which has been shown to be the major haplotype in both healthy and asthmatic populations (274). There are no disease associations with the CAAC CYSLTR1 promoter haplotype but studies are limited and there is a distinct lack of reproducible data that could be due to small sample sizes, the fact that asthma isn't just one disease state (heterogeneity) and potentially geometric differences (268-270).

Sequencing of the coding region again identified that LAD2 and LUVA cells were 100 % identical and a common SNP (SNP 6, rs320995, Table 5.1) was observed replacing the C allele for a T. This SNP is synonymous as the codons TTT and TTC encode the same amino acid, phenylalanine. This means that there would be no differences in the protein structure of $CysLT_1$. However Duan *et al.* have suggested that changes in alleles could alter mRNA translation and stability, even in the case of a synonymous SNP (275). Their study identified two functional synonymous SNPs in the dopamine D2 receptor that altered receptor expression and regulation. Although this is an interesting concept, that synonymous SNPs might not just be "silent" mutations, this unfortunately cannot help to explain why there is differential $CysLT_1$ expression and responses to LTE_4 in LAD2 and LUVA cells, as both regions, promoter and coding, are identical. If genetic variations are not involved in LTE_4's "full" agonist characteristics then some other factors must be contributing to this phenomenon.

Chapter 6

Identification of the requirements for potent LTE_4 signalling in human mast cells

6.1 Introduction

Leukotrienes have been thought to have two modes of action. Primary mechanisms are a direct result of leukotriene stimulation, such as observed in Guinea pig trachea, vascular smooth muscle and human bronchioles. Secondary mechanisms arise from cysteinyl leukotriene-induced activation of arachidonic acid release, which could lead to either leukotriene or prostanoid (prostaglandins, thromboxanes and prostacyclin) production. In guinea pig lungs, LTD_4 has been shown to induce the production of thromboxanes and prostaglandins (276). More recently, LAD2 cells have been shown to robustly produce prostaglandin D_2 (PGD_2) upon stimulation with LTD_4 and LTE_4 (257). Interestingly, in Th2 cells, LTE_4 was able to significantly potentiate PGD_2-induced cytokine production. This suggests that such cysteinyl leukotriene-induced eicosanoid production could have the ability to potentiate responses in an auto/paracrine fashion.

Signalling transduction pathways of $CysLT_1$ have been previously studied but unfortunately, the majority of the studies have focused only on LTD_4 signalling. Another issue that has arisen from previous reports is $CysLT_1$'s G-protein promiscuity (also discussed in section 1.1.4 *Cysteinyl leukotriene receptors*). $CysLT_1$ has the ability to activate several different G-protein associated pathways. Both signalling pathways and GPCR coupling differ hugely from cell to cell.

In isolated human monocytes, LTD_4-induced intracellular calcium mobilisation and gene expression were fully inhibited by MK-571 and pertussis toxin suggesting signalling occurs through $CysLT_1$ coupled to $G\alpha_i$ (134). Intracellular calcium was also inhibited by the IP_3 receptor inhibitor, 2APB, the PLC inhibitor, U73122, calcium chelators BAPTA (intracellular calcium) and EGTA (extracellular calcium), and finally by the thapsigargin, which depletes endoplasmic reticulum stores. This means that LTD_4-induced calcium mobilisation is mediated by PLC and the IP_3 receptor, the activation of store-operated calcium channels and both intracellular and extracellular calcium are required for signalling. Gene expression was inhibited by 2APB and BAPTA but also by p38 and Erk inhibitors. This suggests that LTD_4-induced gene expression in primary monocytes is mediated by $CysLT_1$ in a $G\alpha_i$/calcium/MAPK dependent manner (134).

Interestingly, two other studies have analysed LTD_4 responses in the monocytic cell lines THP-1 and U937 and have identified two distinct $CysLT_1$ signalling pathways; pertussis toxin-sensitive and pertussis toxin-insensitive. Hoshino *et al.* observed ~30 % inhibition of intracellular calcium mobilisation induced by LTD_4 in THP-1 cells by pertussis toxin pretreatment (133). This LTD_4-induced pertussis toxin-sensitive pathway, which implies coupling to $G\alpha_i$, was able to inhibit forskolin-induced cAMP and was also implicated in

chemotaxis. The LTD_4-induced pertussis toxin-insensitive pathway was shown to involve Erk activation. Erk phosphorylation was only slightly sensitive to pertussis toxin and partially inhibited by BAPTA and the pan-PKC inhibitors GF101203X and staurosporine. Complete abrogation of Erk activation was observed by TPA, which depletes endogenous PKCs. This suggests that the pertussis toxin insensitive pathway in THP-1 cells activates Erk in a PKC-dependent pathway that is only partially dependent on calcium.

In differentiated U937 (dU937) cells, these $CysLT_1$ mediated LTD_4-induced pertussis toxin-sensitive and –insensitive pathways were observed but a different signal transduction pathway was suggested (277). The difference stems from the Erk activation. LTD_4-induced Erk phosphorylation is completely inhibited by pertussis toxin in dU937 cells where as it is only slightly inhibited in THP-1 cells (133). This activation of Erk was partially inhibited by *Clostridium sordellii* lethal toxin, which inhibits small GTPases such as Ras, Rap and Rac. LTD_4 was shown to induce Ras activation in these cells and was completely inhibited by pertussis toxin, BAPTA and by an inhibitor of PLC. This suggests that the pertussis toxin-sensitive pathway in dU937 cells, which is mediated by $G\alpha_i$, activates Ras in a calcium-dependent manner which is only partially responsible for Erk activation.

To complicate the picture more, in the intestinal epithelial cell line, Int407, LTD_4-induced Ras activation was shown to be insensitive to pertussis toxin treatment and the $G\alpha_i$ mediated pathway activated Erk through PKC-epsilon and Raf-1. This shows that $CysLT_1$ signal transduction pathways must be highly regulated from cell to cell which could be influenced by constitutive expression levels of these effector molecules and by GPCR regulatory proteins.

6.2 Study Aims

Our previous data strongly suggested that $CysLT_1$ is responsible for cysteinyl leukotriene signalling in the human mast cell lines, LAD2 and LUVA. As there were no genetic variations identified in the CYSLTR1 gene sequence between LAD2 and LUVA cells, other factor/s must be contributing to the potent responses to LTE_4 in LAD2 cells. Most of the previous studies analysing $CysLT_1$ signalling pathways focused on LTD_4, as the major agonist for the receptor, excluding LTE_4 from the analysis (as a final leukotriene metabolite and partial agonist) thus any differences between LTD_4 and LTE_4 signalling are poorly understood.

The aim of this study is to elucidate the signalling requirements that allow LTE_4 to act as a "full" agonist in LAD2 cells. LTD_4 and LTE_4 signalling pathways will be compared between LAD2 and LUVA cells in order to identify cell specific requirements for potent responses to LTE_4.

6.3 Results

6.3.1 Comparison of CYSLTR1 and CYSLTR2 in human mast cell lines LAD2 and LUVA

Our initial results already suggested that CYSLTR1 is differentially expressed in LAD2 and LUVA cells. Looking more comprehensively at the microarray data, CYSLTR1 was consistently more highly expressed in LAD2 cells than in LUVA, while CYSLTR2 expression levels were very low and similar in both cell lines (Figure 6.1A-B). qRT-PCR also confirmed that LAD2 cells have higher expression of CYSLTR1 mRNA than LUVA cells (Figure 6.2A). Generally both cell lines have a much higher expression level of CYSLTR1 than CYSLTR2 and 2 hour stimulations with LTD_4 or LTE_4 had no effect on the expression of either receptor.

To ascertain whether the expression level of $CysLT_1$ is relevant for mast cells responsiveness to cysteinyl leukotrienes, $CysLT_1$ was overexpressed in LUVA cells. Forward constructs of human $CysLT_1$ tagged with 3xHA and vectors without the gene (Empty controls) were stably transfected into LUVA cells using lentiviral transduction and positive clones selected using puromycin. qRT-PCR confirmed a 3-fold increase in CYSLTR1 expression in the [$CysLT_1$-3xHA] transfectant population (Figure 6.2B) but unfortunately when using an antibody against the HA tag, no increase in fluorescence was observed compared to the control cells, suggesting lack of receptor surface expression (Figure 6.2C). Stimulation for 2 hours with either LTD_4 or LTE_4 revealed no differences in

responses to both leukotrienes, measured as CCL4 mRNA between $CysLT_1$ transfectants and empty control cells (Figure 6.2D).

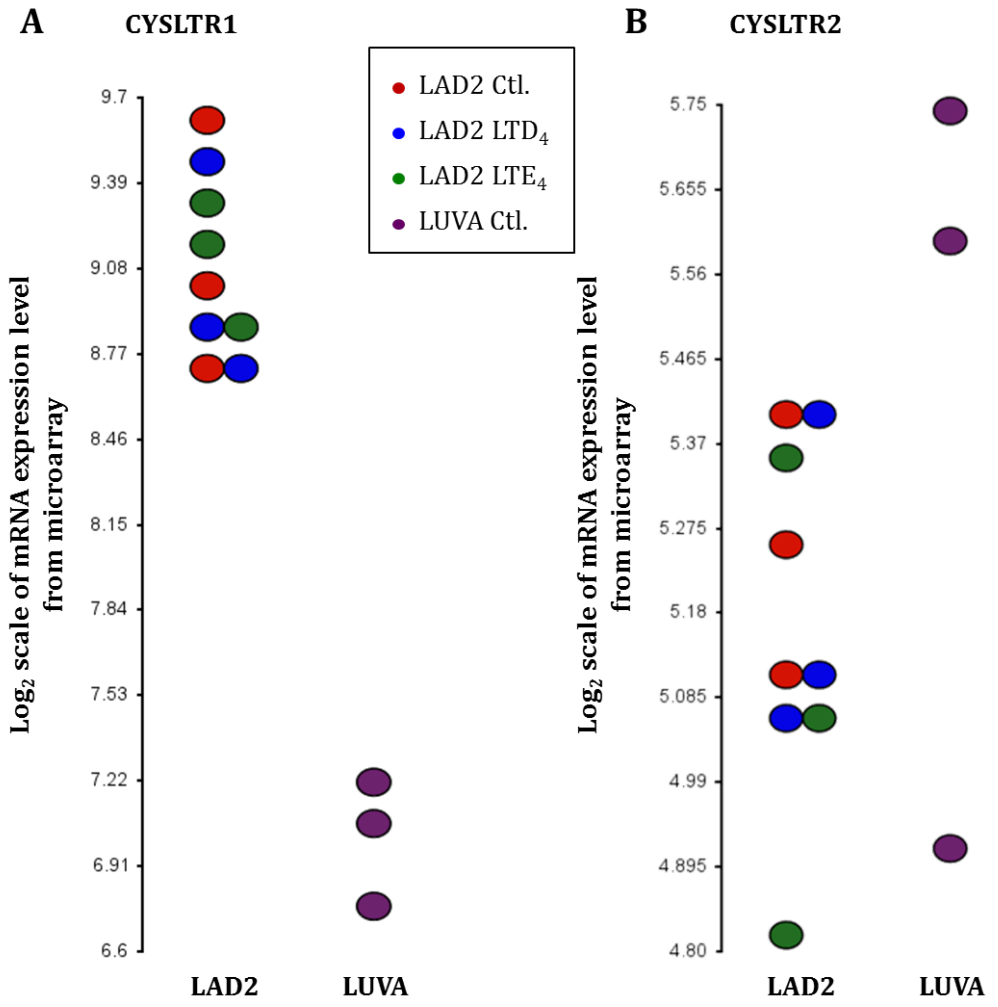

Figure 6.1 Comparison of CYSLTR1 and CYSLTR2 expression levels between LAD2 and LUVA cells.

(A) CYSLTR1 and **(B)** CYSLTR2 mRNA expression levels in LAD2 and LUVA cells stimulated for 2 hours in the presence of L-cysteine 3 mmol/L with either vehicle control (Ctl.), LTD_4 100 nmol/L or LTE_4 100 nmol/L analysed using Affymetrix Human Gene 1.0 ST arrays, data expressed as log_2 of expression intensity values.

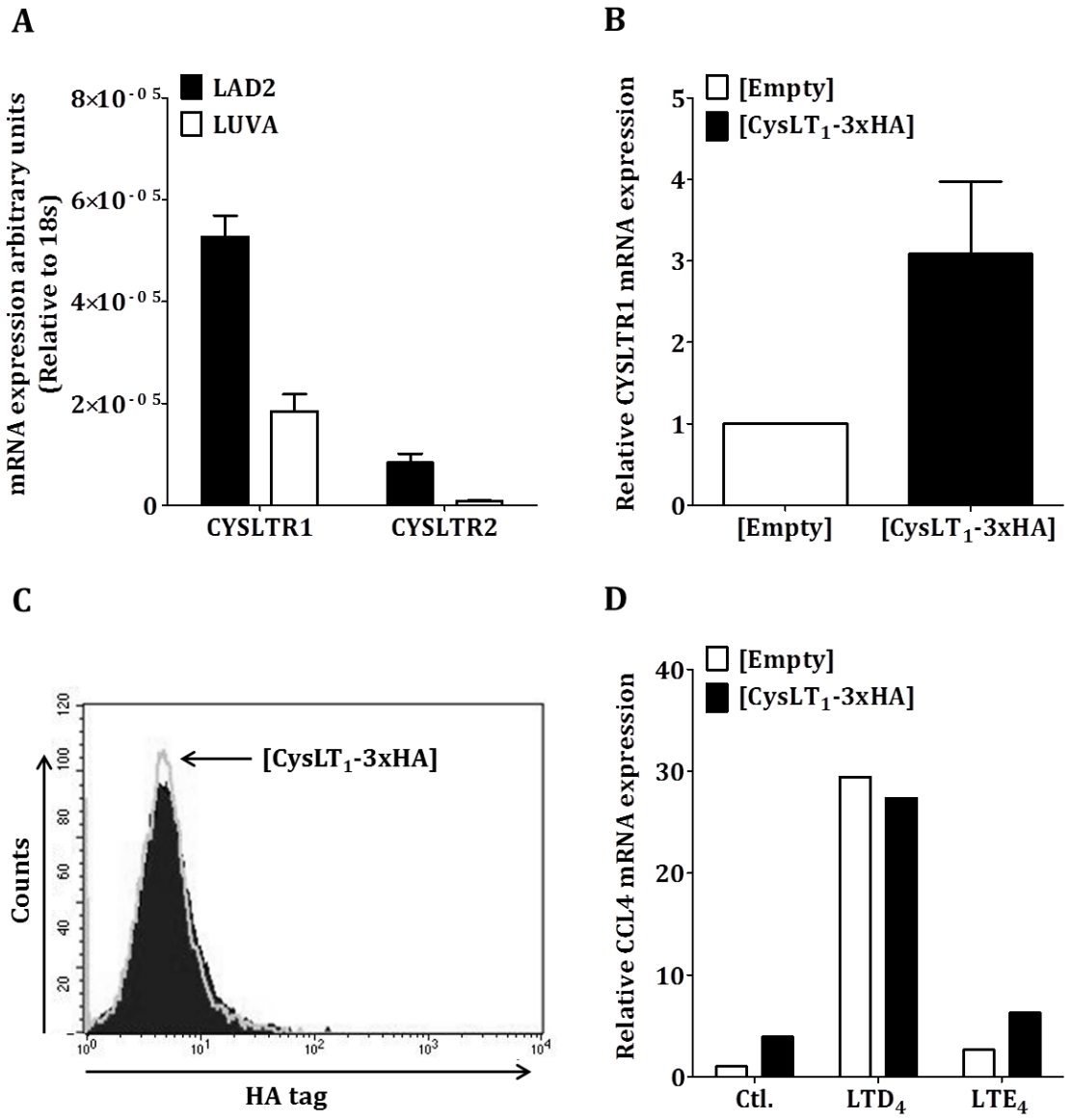

Figure 6.2 The effect of $CysLT_1$ overexpression in LUVA cells.

(A) mRNA expression level of CYSLTR1 and CYSLTR2 in LAD2 and LUVA cells measured by qRT-PCR, *n*=3, 18s used as endogenous control, data expressed as arbitrary units. Forward constructs of 3xHA tagged $CysLT_1$ were stably transfected into LUVA cells using lentivirus, **(B)** relative quantification of CYSLTR1 mRNA in [$CysLT_1$-3xHA] transfectants, data relative to [Empty] control, 18s used as endogenous control, *n*=3, **(C)** Flow cytometric analysis of $CysLT_1$ expression in [$CysLT_1$-3xHA] (grey line) and [Empty] (filled black) transfectants using a Alexa Fluor labelled antibody specific for 3xHA, 1:1000, results from a single experiment representative of 3, **(D)** relative quantification of CCL4 mRNA in [Empty] (open bars) and [$CysLT_1$-3xHA] (closed bars) transfectants stimulated for 2 hours in the presence of L-cysteine 3 mmol/L with either LTD_4 100 nmol/L, LTE_4 100 nmol/L or vehicle control (Ctl.), data relative to Ctl., 18s used as endogenous control, one experiment run in triplicate. Data in **(A)** and **(B)** represented as mean ± SEM.

6.3.2 Analysis of CysLT$_1$ G-protein coupling in human mast cell lines

As previously stated, CysLT$_1$ has the potential to signal via two different Gα proteins, Gα$_i$ and Gα$_q$, and so it is important to first ascertain how CysLT$_1$ couples in both mast cell lines. Pertussis toxin (Ptx) is a protein that specifically catalyses the ADP-ribosylation of Gα$_i$, therefore inhibiting the G-protein's ability to bind to GPCRs and thus is an invaluable tool for differentiating signalling pathways (61,73). Cysteinyl leukotrienes induce robust intracellular calcium fluxes in both LAD2 and LUVA cells (Figure 4.3). Intracellular calcium mobilisation can be induced by GPCRs coupled to either Gα$_q$ or Gα$_i$ so responses were analysed in both cell lines when pretreated with pertussis toxin. LTC$_4$, LTD$_4$ and LTE$_4$ induced a robust concentration-dependent calcium mobilisation in LAD2 cells that was completely unaffected by pertussis toxin pretreatment (Figure 6.3A). This lack of sensitivity to pertussis toxin treatment was also observed in LUVA cells where the concentration-dependent calcium fluxes were unaltered suggesting that calcium responses are exclusively Gα$_q$ mediated in both these cell lines (Figure 6.3B). The effect of pertussis toxin treatment on gene expression was also analysed. Looking at COX2 and CCL4 mRNA expression, pertussis toxin treatment did not affect LTD$_4$ or LTE$_4$-induced responses in both LAD2 and LUVA cells (Figure 6.4). This suggests that CysLT$_1$ signalling pathways that lead to gene expression are Gα$_q$ mediated in both cell lines.

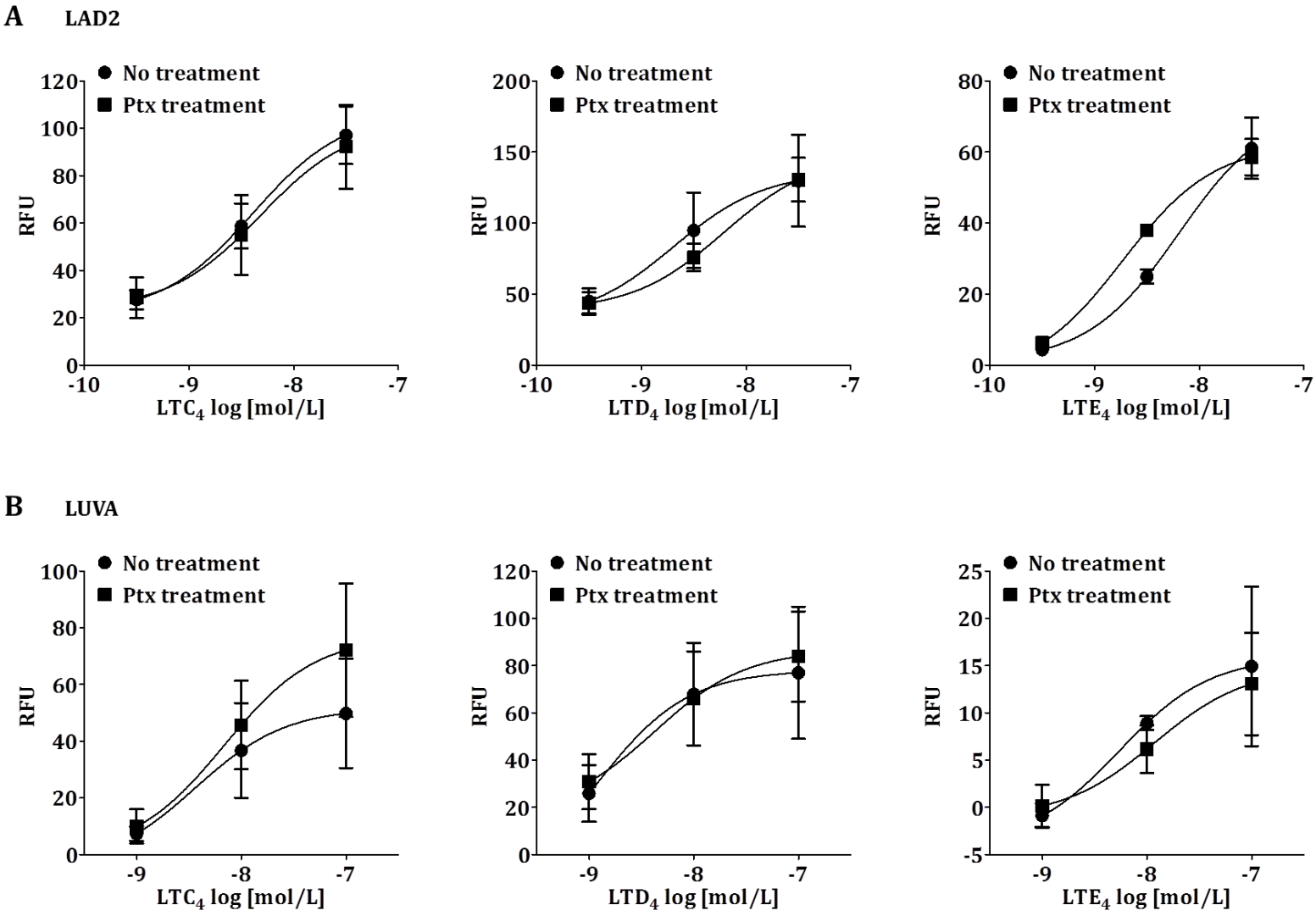

Figure 6.3 Cysteinyl leukotriene-induced intracellular calcium mobilisation is not mediated by $G\alpha_i$ in human mast cell lines.
Baseline corrected peak calcium flux data of **(A)** LAD2 and **(B)** LUVA cells stimulated with the indicated concentrations of either LTC_4, LTD_4 or LTE_4 with or without an overnight preincubation with pertussis toxin (Ptx) 100 ng/ml, *n*=3 run in triplicate. Data represented as mean ± SEM. Relative fluorescence units (RFU).

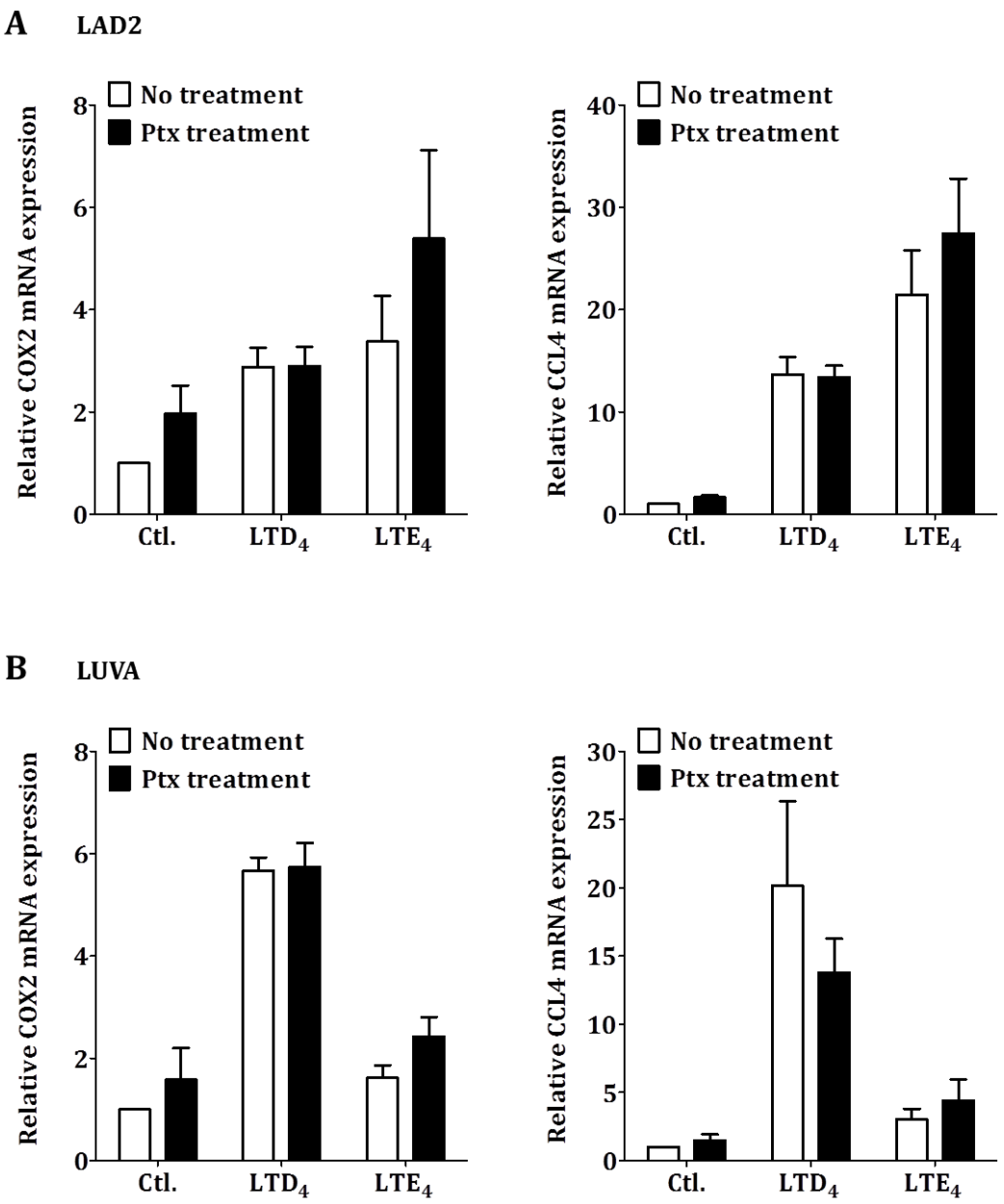

Figure 6.4 Cysteinyl leukotriene-induced gene expression is not mediated by $G\alpha_i$ in human mast cell lines.

Relative quantification of COX2 and CCL4 expression in **(A)** LAD2 and **(B)** LUVA cells stimulated for 2 hours with either LTD_4 100 nmol/L, LTE_4 100 nmol/L or vehicle control (Ctl.) in the presence of L-cysteine 3 mmol/L with or without overnight pertussis toxin (Ptx) 100 ng/ml preincubation, data relative to untreated Ctl. 18s used as endogenous control, *n*=3. Data represented as mean ± SEM.

6.3.3 Analysis of potential modifiers of the cysteinyl leukotriene signalling pathway

Cyclooxygenases (COX1 or COX2) are key enzymes involved in the production of prostaglandins (278). As previously stated, a cross talk of leukotriene and prostaglandin pathways has been hypothesised for many years as a potential way to potentiate responses. To ascertain whether this could be the cause of the potent responses to LTE_4 in LAD2 cells, relative quantification of CCL4 mRNA expression was analysed in response to cysteinyl leukotriene stimulation with or without pretreatment with specific inhibitors. Indomethacin, a nonselective inhibitor of cyclooxygenase, and NS-398 a selective COX2 inhibitor had no effect on LTE_4 or LTD_4-induced responses in LAD2 cells (Figure 6.5A). This lack of response attenuation was mirrored in LUVA cells (Figure 6.5B). To analyse whether $CRTH_2$ (a receptor for prostaglandin D2 expressed on mast cells) plays a role in these responses, the specific inhibitor CAY10471 was used. LTD_4 and LTE_4-induced mRNA expression in both LAD2 and LUVA cells were unaffected by inhibiting $CRTH_2$ (Figure 6.5A-B).

Previous reports have suggested that PPAR-γ is involved in the leukotriene signalling pathway in mast cells, almost exclusively in LTE_4-induced responses (257). To analyse whether cysteinyl leukotrienes responses are mediated by PPAR-γ in either LAD2 or LUVA cells, relative quantification of CCL4 mRNA was measured with or without a pretreatment with the PPAR-γ inhibitor, GW9662. Surprisingly, GW9662 had no effect on LTD_4 or LTE_4-induced CCL4 mRNA expression in LAD2 cells, contradictory to what has been reported (Figure 6.5C)(257). This was also mimicked in the LUVA cells (Figure 6.5D). To confirm these findings another PPAR-γ inhibitor, T0070907, was tested. Pretreatment of LAD2 and LUVA cells with T0070907 again had no effect on LTD_4 or LTE_4-induced CCL4 mRNA expression (Figure 6.5C-D).

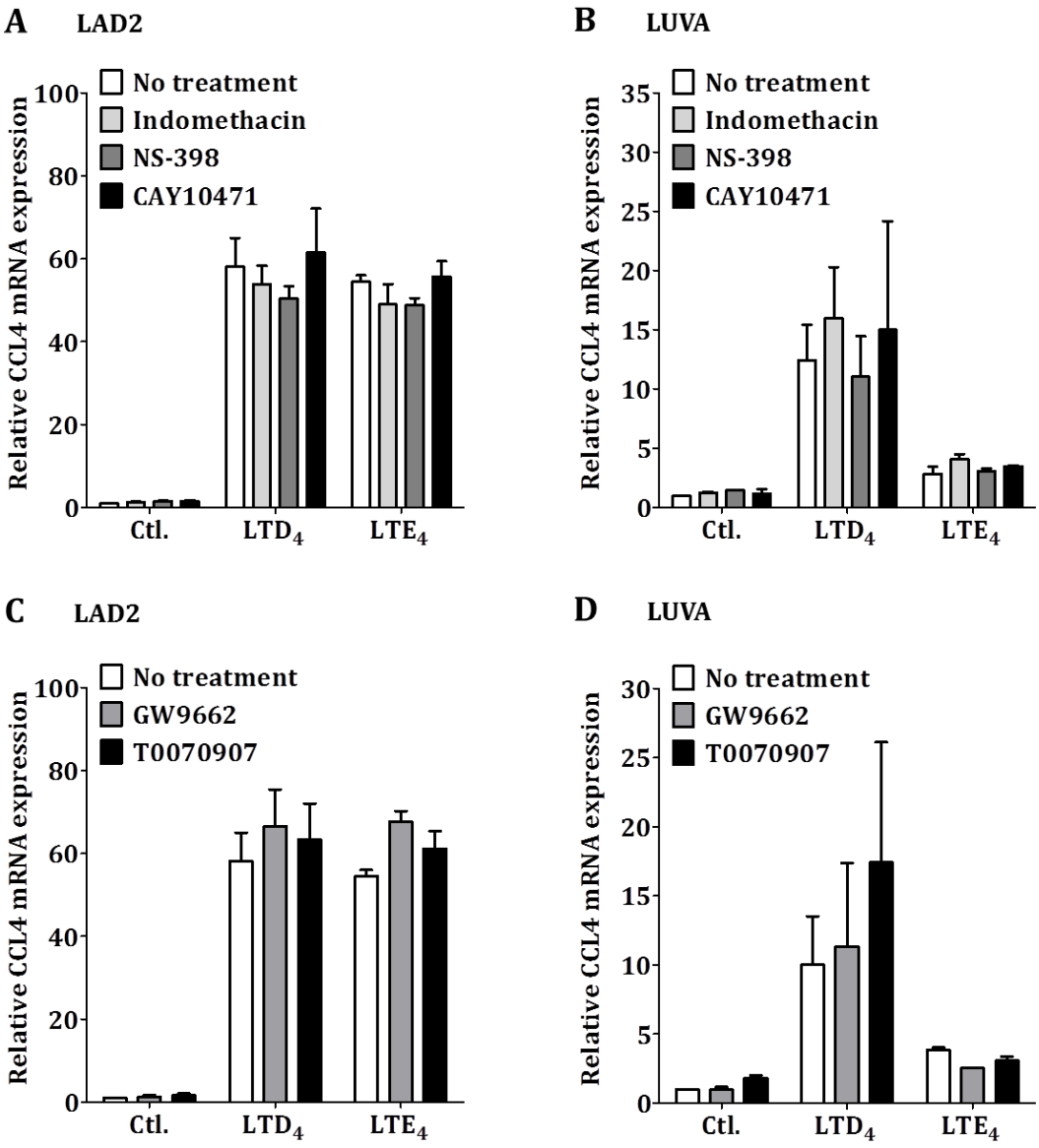

Figure 6.5 The effect of COX, $CRTH_2$ and PPAR-γ inhibition on cysteinyl leukotriene responses in mast cell lines.

Relative quantification of CCL4 mRNA expression in the indicated mast cell lines stimulated with either LTD_4 100 nmol/L, LTE_4 100 nmol/L or vehicle control (Ctl.) for 2 hours in the presence of L-cysteine 3 mmol/L with or without the pretreatment of either **(A-B)** indomethacin 10 µmol/L for 30 minutes, NS-398 10 µmol/L for 30 minutes or CAY10471 100 nmol/L for 10 minutes, *n*=2-4 **(C-D)** GW9662 10 µmol/L or T0070907 1 µmol/L for 1 hour, *n*=2. Data relative to untreated Ctl. 18s used as endogenous control. Data represented as mean ± SEM.

6.3.4 Analysis of cysteinyl leukotriene signalling pathways in LAD2 and LUVA cells

As intracellular calcium mobilisation and gene expression is mediated by a $G\alpha_q$ coupled $CysLT_1$ receptor in both LAD2 and LUVA cells, it is important to ascertain whether there are any differences in downstream signalling pathways that might explain the potency of LTE_4 in LAD2 cells. Pretreatment of both mast cell lines with the intracellular and extracellular calcium chelators, BAPTA-AM and EDTA, fully abrogated LTD_4 and LTE_4-induced CCL4 expression, suggesting that calcium is essential for gene expression to occur (Figure 6.6A-B). Inhibition of JNK had no effect on cysteinyl leukotriene responses in either cell line (Figure 6.6C-D). PI3K inhibition surprisingly did not affect LTD_4 responses in LAD2 cells, as this has been previously reported, but did inhibit CCL4 expression induced by LTD_4 in LUVA cells (Figure 6.7A-B)(257). Responses to LTE_4 were insensitive to PI3K inhibition in both mast cell lines. Pretreatment of LAD2 and LUVA cells with the MEK inhibitor (Erk pathway) U-0126 significantly inhibited LTD_4 and LTE_4-induced CCL4 expression (Figure 6.7C-D).

Cysteinyl leukotriene-induced CCL4 expression in LAD2 cells was also affected by pretreatment with the pan-PKC inhibitor Gö 6983 (Figure 6.8A). This partial inhibition of leukotriene-induced responses was not observed when analysing intracellular calcium mobilisation (Figure 6.8C) and in LUVA cells, where all responses, gene expression and intracellular calcium mobilisation were completely insensitive to PKC inhibition (Figure 6.8B and D). Microarray data of both cell lines comparing PKC isozymes revealed substantial differences in expression levels of PKC alpha, beta, epsilon and eta that could potentially attribute to the difference observed (Figure 6.8E).

This means that in both cell lines $CysLT_1$-$G\alpha_q$ mediated gene expression requires both intracellular and extracellular calcium and is dependent on Erk activation. Gene expression is not dependent on the activation of COX, $CRTH_2$, PPAR-γ or JNK. In LAD2 cells, LTD_4 and LTE_4-induced gene expression is partially dependent on the activation of a calcium-independent PKC. While in LUVA cells, gene expression is not dependent on activation of PKC. Only LTD_4-induced gene expression in LUVA cells seems to require the activation of PI3K.

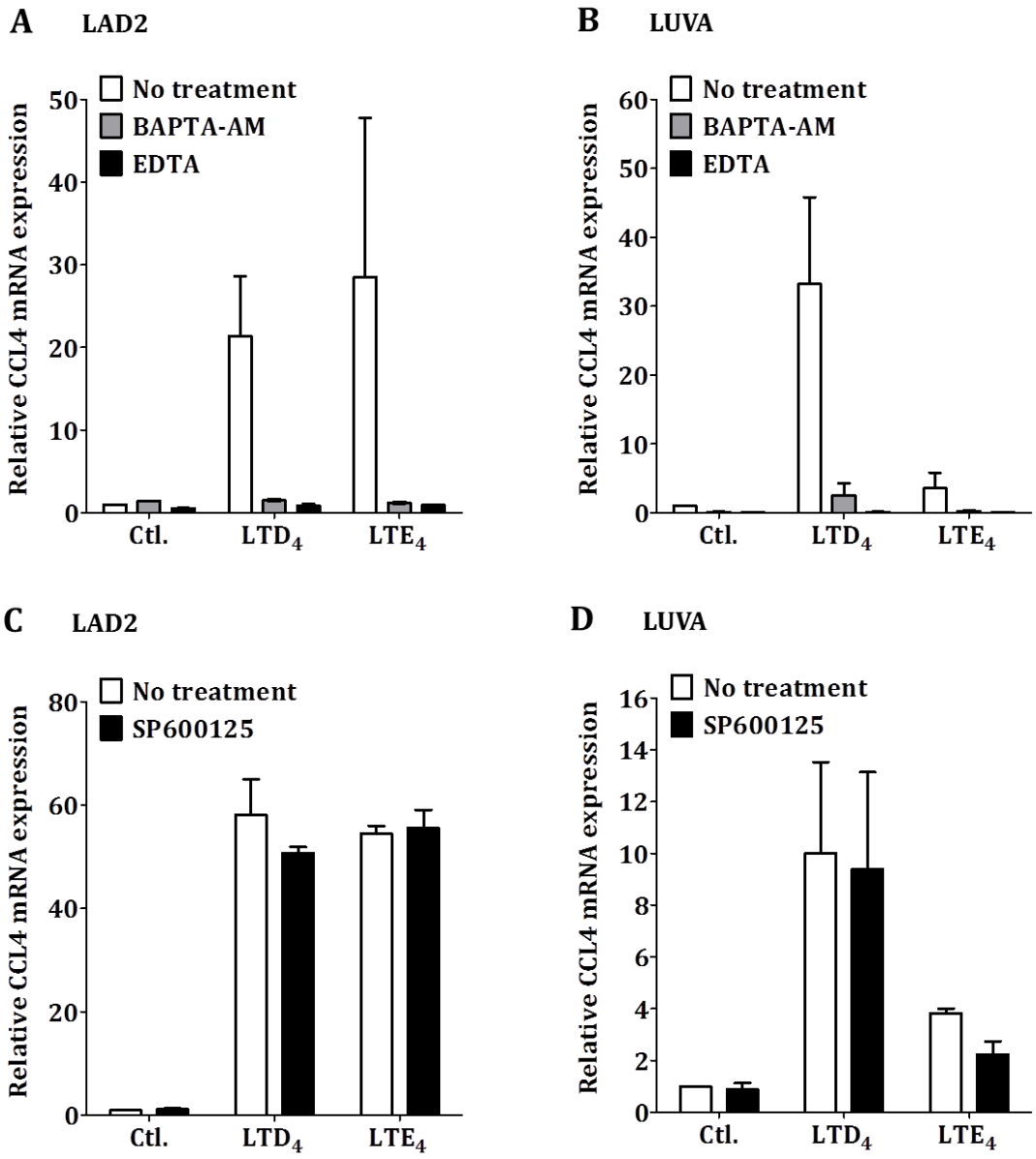

Figure 6.6 The effect of calcium depletion and JNK inhibition on cysteinyl leukotriene responses in mast cell lines.

Relative quantification of CCL4 mRNA expression in the indicated mast cell lines stimulated with either LTD_4 100 nmol/L, LTE_4 100 nmol/L or vehicle control (Ctl.) for 2 hours in the presence of L-cysteine 3 mmol/L with or without the pretreatment of either **(A-B)** BAPTA-AM 30 µmol/L for 30 minutes or EDTA for 10 minutes, *n*=3 **(C-D)** SP600125 1 µmol/L for 1 hour, *n*=2. Data relative to untreated Ctl. 18s used as endogenous control. Data represented as mean ± SEM.

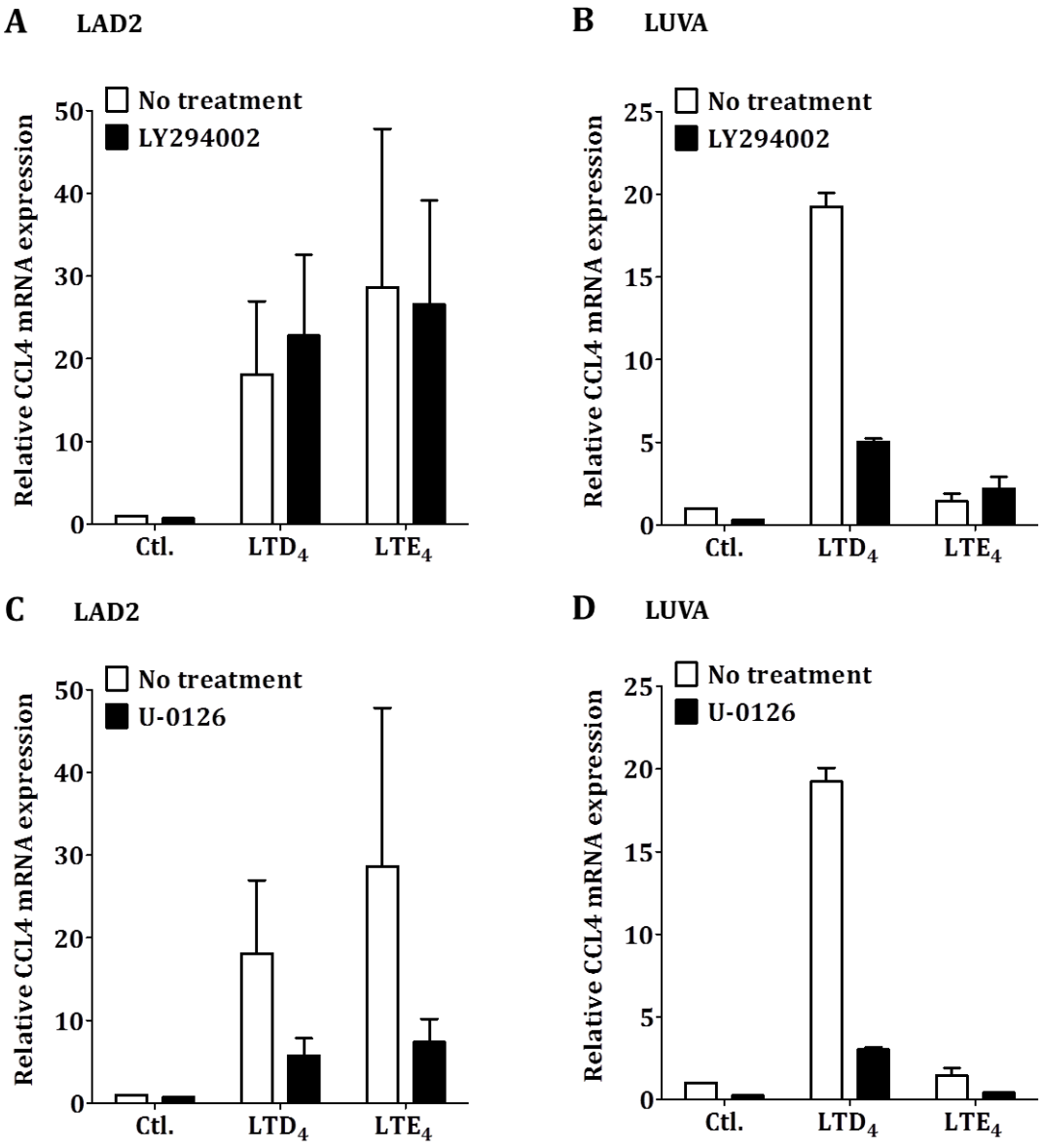

Figure 6.7 The effect of PI3K and MEK inhibition on cysteinyl leukotriene responses in mast cell lines.

Relative quantification of CCL4 mRNA expression in the indicated mast cell lines stimulated with either LTD_4 100 nmol/L, LTE_4 100 nmol/L or vehicle control (Ctl.) for 2 hours in the presence of L-cysteine 3 mmol/L with or without the pretreatment of either **(A-B)** LY294002 10 µmol/L for 30 minutes, *n*=3 **(C-D)** U-0126 1 µmol/L for 30 minutes, *n*=3. Data relative to untreated Ctl. 18s used as endogenous control. Data represented as mean ± SEM.

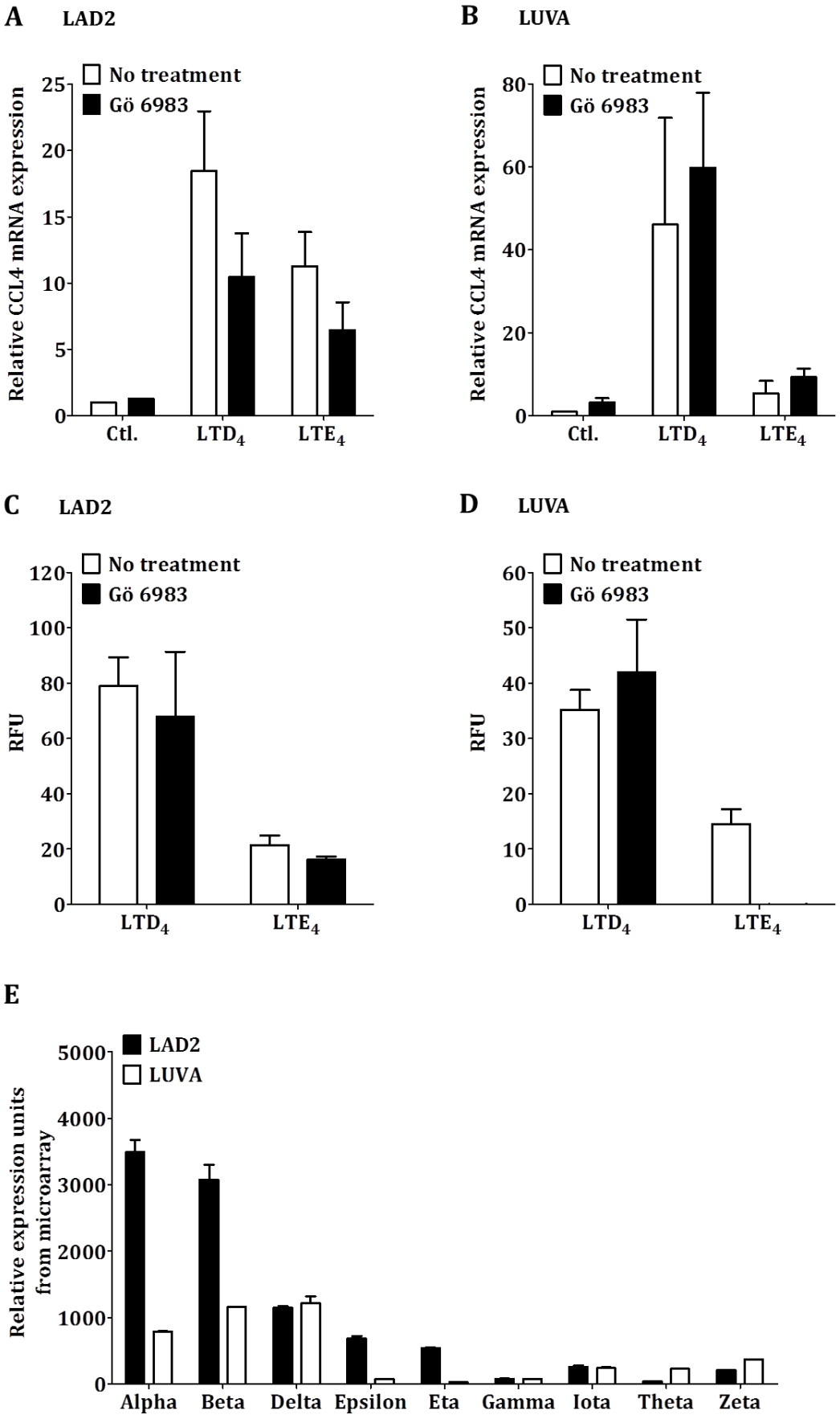

Figure 6.8 The effect of PKC inhibition on cysteinyl leukotriene responses in mast cell lines.

Relative quantification of CCL4 mRNA expression in **(A)** LAD2 and **(B)** LUVA cells stimulated with either LTD_4 100 nmol/L, LTE_4 100 nmol/L or vehicle control (Ctl.) for 2 hours in the presence of L-cysteine 3 mmol/L with or without a 30 minute pretreatment with Gö 6983 1 μmol/L, *n*=2, data relative to untreated Ctl. 18s used as endogenous control. Baseline corrected peak calcium flux data of **(C)** LAD2 and **(D)** LUVA cells stimulated with either LTD_4 100 nmol/L or LTE_4 100 nmol/L with or without a 30 minutes pretreatment with Gö 6983 1 μmol/L, *n*=2-3. **(E)** Relative expression of PKC isozymes in LAD2 (closed bar) and LUVA (open bar) cells analysed using Affymetrix microarrays, *n*=3. Data represented as mean intensity values ± SEM. Relative fluorescence units (RFU).

6.3.5 Analysis of cysteinyl leukotrienes agonist dynamics

Since there were no differences identified in signalling pathways between LTD_4 and LTE_4 in LAD2 cells there must be another explanation to why LTE_4 is a potent agonist in this model and not in any other cell models analysed such as our overexpression models and LUVA cells. In previous experiments it was shown that cysteinyl leukotriene-induced gene expression is partially dependent on Erk activation (Figure 6.7C-F). To further analyse the agonist actions of LTE_4, time course experiments of Erk phosphorylation were conducted and analysed by Western blot. In LAD2 cells stimulated with LTD_4, Erk phosphorylation peaked at 7 minutes with a gradual decrease until 60 minutes where detected Erk phosphorylation was very weak (Figure 6.9A). LTE_4 induced a peak of Erk phosphorylation at around 10-15 minutes with a more sustained phosphorylation, still being detectable at the 60 minutes time point. In LUVA cells the point at which Erk phosphorylation peaked was very similar to LAD2 cells (LTD_4 ~ 5-7 minutes and LTE_4 ~ 10-15 minutes, Figure 6.9B). LTE_4-induced Erk phosphorylation in LUVA cells was far less prolonged than in LAD2 cells with detected phosphorylation being similar to that of the control at 60 minutes (Figure 6.9B).

To analyse how the prolonged signalling caused by LTE_4 in LAD2 cells could potentially effect gene expression, long and short ligand exposure experiments were set up and CCL4 mRNA expression was analysed. For the short ligand exposure, cells were stimulated with either LTD_4 or LTE_4 for 5 minutes, washed and then lysed for mRNA analysis after a 2 hour (total) incubation. For the long ligand exposure, cells were stimulated for 2 hours (continuously) with either LTD_4 or LTE_4 then lysed for mRNA analysis. In LAD2 cells, the 2 hour exposure revealed similar results to what has been seen previously, the response to LTE_4 was 97 % of LTD_4(mean, Figure 4.2A and Figure 6.9C). In the 5 minute exposure, the overall induction of CCL4 mRNA was much lower but interestingly, the response to LTE_4

was much weaker than LTD_4 with LTE_4 being able to reach only 32 % of the response to LTD_4 (mean). In LUVA cells, the order of ligand potencies remained the same between the two exposure times: LTE_4 was far less potent than LTD_4 (31 % and 13 %, mean, 2 hours and 5 minutes respectively; Figure 6.9D).

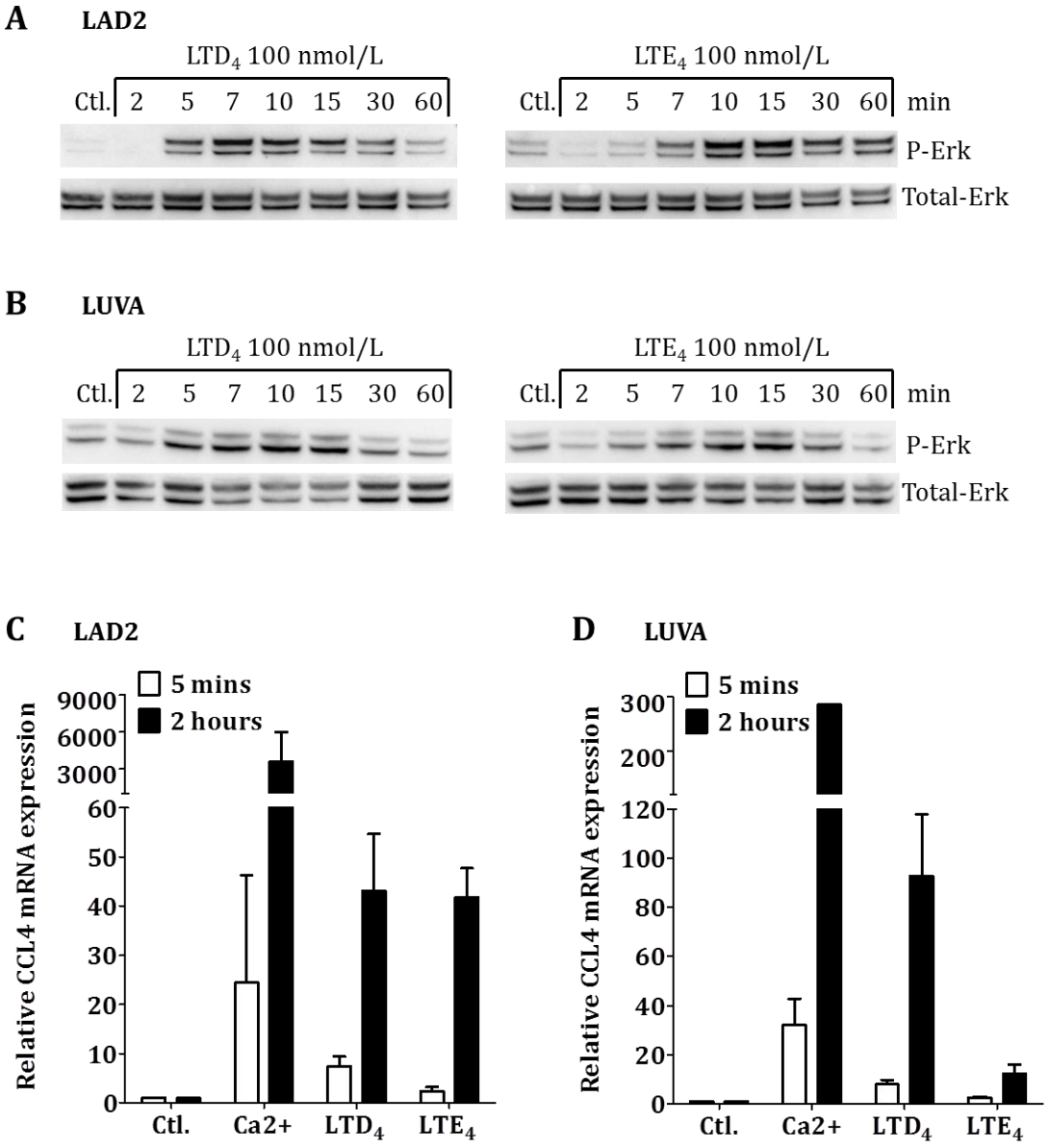

Figure 6.9 The effect of cysteinyl leukotrienes dynamics in human mast cell lines.
Western blot analysis of cell lysates obtained from either **(A)** LAD2 or **(B)** LUVA cells stimulated for the indicated times with either vehicle control (Ctl.), LTD_4 100 nmol/L or LTE_4 100 nmol/L using antibodies specific for phosphorylated (P) Erk and total Erk, results from a single experiment representative of 2. Relative quantification of CCL4 mRNA expression in **(C)** LAD2 and **(D)** LUVA cells exposed, in the presence of L-cysteine 3 mmol/L, for either 5 minutes (open bar) or 2 hours (closed bar) to either vehicle control, calcium ionophore A23187 (Ca2+), LTD_4 100 nmol/L or LTE_4 100 nmol/L for a total

incubation period of 2 hours, data relative to Ctl., 18s used as endogenous control, **(C)** *n*=3 **(D)** *n*=2. Data represented as mean ± SEM.

An important regulatory feature of GPCRs is receptor desensitisation. As previously described, this mechanism allows essentially the termination of signalling through certain pathways such as receptor phosphorylation and receptor internalisation. To ascertain whether each cysteinyl leukotriene could induce receptor desensitisation, stimulations were performed at 20 and 120 seconds on the same cell population and intracellular calcium mobilisation was analysed. In LAD2 cells, all three cysteinyl leukotrienes induced a robust flux in intracellular calcium in the initial 20 second stimulation (Figure 6.10A-C). The calcium response to LTD_4 was completely abrogated by prior stimulation with either LTC_4 or LTD_4, suggesting that both LTC_4 and LTD_4 can fully desensitise $CysLT_1$ in LAD2 cells (Figure 6.10A-B). Interestingly, the calcium response to LTD_4 was only partially inhibited by prior stimulation with LTE_4 showing that LTE_4 can only partially desensitise $CysLT_1$ (Figure 6.10C). In LUVA cells, a similar pattern of ability to desensitise was exhibited. LTC_4 and LTD_4 were able to fully abrogate the second stimulation by LTD_4 while LTE_4 was unable/partially inhibited the calcium response to LTD_4 (Figure 6.11A-C).

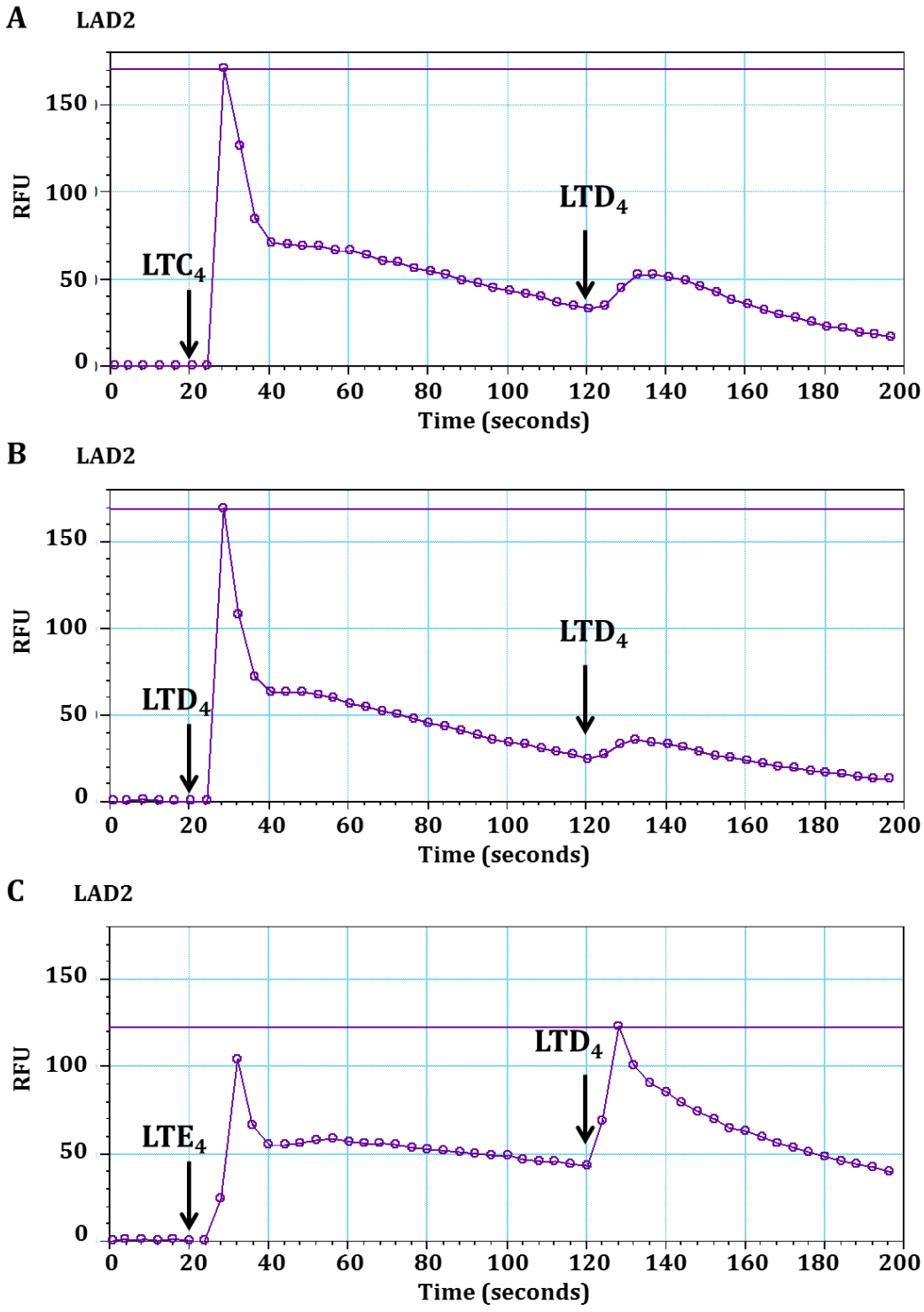

Figure 6.10 The effect of cysteinyl leukotrienes on receptor desensitisation in LAD2 cells.

Raw calcium flux data of LAD2 cells stimulated at 20 and 120 seconds with 100 nmol/L of **(A)** LTC_4 then LTD_4, **(B)** LTD_4 then LTD_4, **(C)** LTE_4 then LTD_4, representative of 3 experiments run in triplicate, relative fluorescence units (RFU), black arrows indicates start of stimulation.

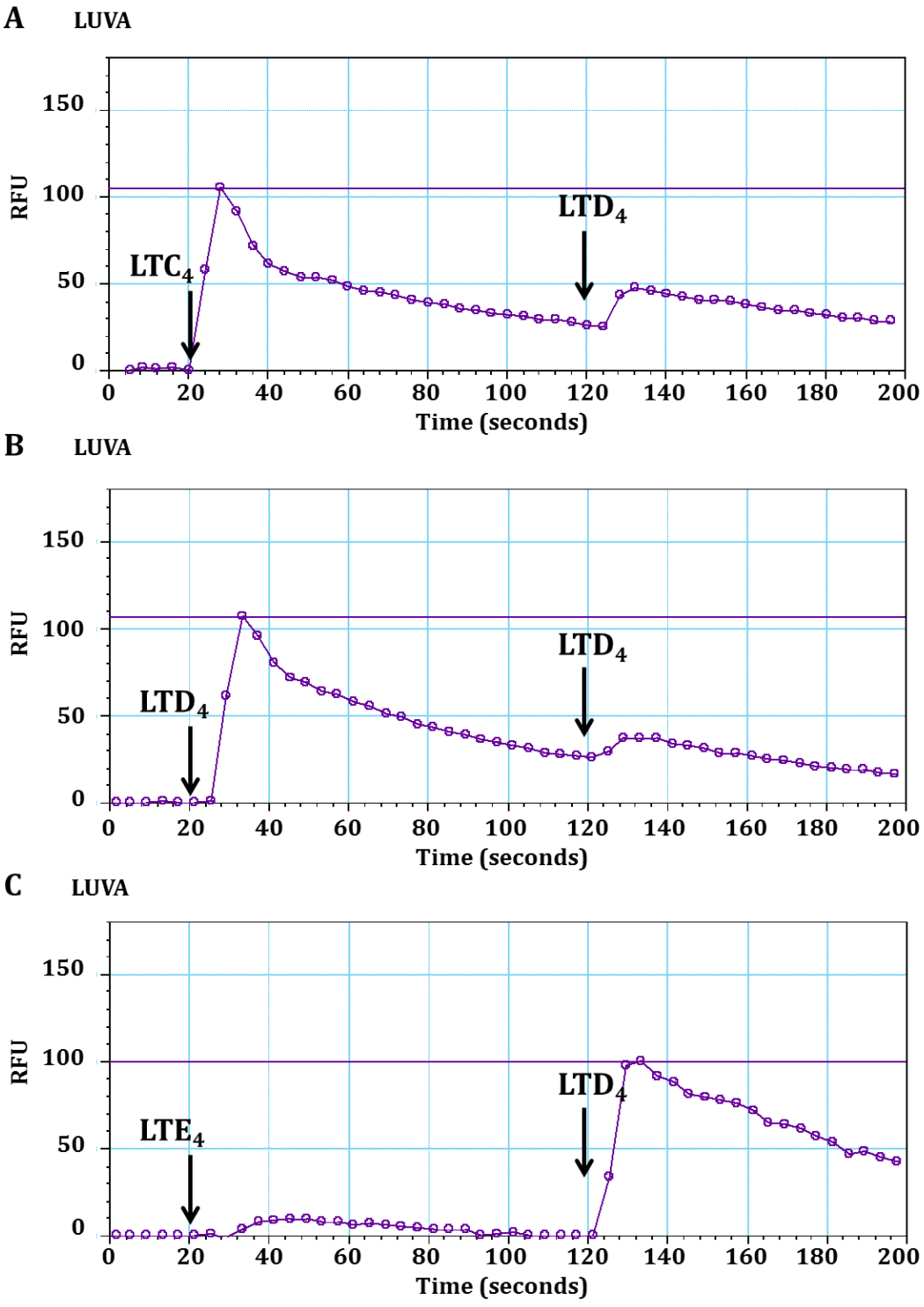

Figure 6.11 The effect of cysteinyl leukotrienes on receptor desensitisation in LUVA cells.

Raw calcium flux data of LUVA cells stimulated at 20 and 120 seconds with 100 nmol/L of **(A)** LTC_4 then LTD_4, **(B)** LTD_4 then LTD_4, **(C)** LTE_4 then LTD_4, representative of 3 experiments run in triplicate, relative fluorescence units (RFU), black arrows indicates start of stimulation.

6.4 Discussion

Comparison of GPCR expression levels in LAD2 and LUVA cells showed higher constitutive $CysLT_1$ mRNA expression in LAD2 cells. This finding was also confirmed by qRT-PCR (Figure 6.1 and Figure 6.2A). To ascertain whether the amount of expression of the $CysLT_1$ receptor plays an important role in the responses to LTE_4, HA tagged $CysLT_1$ was overexpressed in LUVA cells to see if the potency of LTE_4 could be increased (Figure 6.2B-D). Although an increase in CYSLTR1 was observed at an mRNA level, there was no change in HA expression detected on the cell surface compared to the [Empty] control. As mRNA expression doesn't necessary relate to protein surface expression and due to the lack of reliable $CysLT_1$ antibodies for flow cytometry our aim was to measure receptor surface expression using an antibody to the N-terminal tag peptide. Compared to other tags such as GFP, HA is a relatively small peptide which has been used extensively in previous studies (Chapter 3) and so it was disappointing that the HA-tagged receptor could not be detected on the cell surface (279). $CysLT_1$ itself is well known to be difficult to overexpress in cell systems so this coupled with an N-terminal tag might have significantly affected successful protein folding and/or surface expression. The possibility that a very low number of recombinant $CysLT_1$ molecules were present on the cell surface but not detected due to sensitivity of the assay cannot also be excluded. Unfortunately no conclusion can be made at this stage from this overexpression model. Further experiments need to be carried out, potentially using different expression vectors or an untagged version of $CysLT_1$ in LUVA cells, although with the lack of good antibodies, assessing surface expression will be difficult.

Responses of both cell lines to cysteinyl leukotrienes were unaffected by pertussis toxin treatment revealing that gene expression is mediated by $CysLT_1$ that couples mainly to $G\alpha_q$ in these cells (Figure 6.3 and Figure 6.4). As previously stated, $CysLT_1$ is a GPCR that has the ability to couple to different G-proteins which seems to be cell type dependent. For example, in overexpression models using cRNA injected *Xenupus laevis* oocytes and transfected HEK293 cells, LTD_4-induced responses were not inhibited by pertussis toxin treatment suggesting a $G\alpha_q$ coupled receptor (114,239). $CysLT_1$ responses in Th2 cells and the monocytic cell lines THP-1 and dU937 show partial pertussis toxin sensitivity i.e. $CysLT_1$ coupling to $G\alpha_i$ and $G\alpha_q$ (130,133,277). Eosinophil and monocyte responses to cysteinyl leukotrienes are completely inhibited by pertussis toxin i.e. $CysLT_1$ couples to $G\alpha_i$ in those cells and in alveolar macrophages leukotriene mediated responses are unaffected by pertussis toxin treatment (134,280,281). In human mast cells, LTD4 mediated proliferation has been shown to be mediated by a $G\alpha_i$ coupled $CysLT_1$ receptor (254).

For LTE_4 to be a potent agonist in LAD2 cells and no other cell type previously analysed it was hypothesised that there must be differences in how LTE_4 signals compared to LTD_4. COX and $CRTH_2$ inhibition had no effect on cysteinyl leukotriene-induced gene expression which suggests initial responses activated by cysteinyl leukotrienes only are being observed (Figure 6.5A-B), however this does not rule out LTE_4 activating such secondary mechanisms, as shown by the induction of COX2 mRNA expression (Figure 4.2A) and by PGD_2 release (257). PPAR-γ inhibition also had no effect on gene expression. In Paruchuri *et al.*'s study, LTE_4-induced responses were almost exclusively liable to PPAR-γ inhibition by GW9662 (257). As our results were contradictory to their findings a second PPAR-γ inhibitor, T0070907, was also analysed confirming our initial observations (Figure 6.5).

It has been reported in many studies that LTE_4 is much weaker activator of intracellular calcium mobilisation than LTD_4 (170,239,257,282). Calcium flux is essential for gene expression in both mast cell lines (Figure 6.6A-B) which might explain why the LTE_4-induced upregulation of genes such as CCL4 or GMCSF in LUVA cells is not observed, since the calcium signalling threshold might not have been reached (Figure 4.2B). LTE_4- induced calcium in LAD2 cells was almost half that of LTD_4's but at a gene expression level, responses to LTE_4 matched responses to LTD_4, suggesting additional signalling mechanisms being involved. Analysis of potential leukotriene-induced signalling pathways revealed no differences between LTD_4 and LTE_4 signalling in LAD2 cells. Gene expression was partially affected by inhibitors specific for the MEK (Erk) and the PKC pathways (Figure 6.7C-D and Figure 6.8). Calcium mobilisation was unaffected by pan-PKC inhibition, revealing a signalling pathway independent of calcium mobilisation. As previously stated, PKC isozymes have been classified by their signalling requirements. As PKC inhibition did not affect calcium mobilisation, isozymes from either novel or atypical classification groups i.e. delta, epsilon, eta, mu, theta, lambda or zeta, are potential candidates for mediating cysteinyl leukotriene-induced gene expression in LAD2 cells.

GPCR signalling is highly regulated by constitutive expression levels of a plethora of signalling components such as surface proteins, G-proteins and PKCs. As PKC inhibition did not affect cysteinyl leukotriene responses in LUVA cells, the expression levels of the PKC isozymes between the two mast cell lines was compared (Figure 6.8E). Interestingly, out of the novel and atypical PKCs only epsilon and eta were quite highly differentially expressed (epsilon: LAD2 685, LUVA 72; eta: LAD2 543, LUVA 31, mean, relative expression units). Previous studies have linked PKC-epsilon with cysteinyl leukotriene-induced responses. In intestinal epithelium it has been shown that responses to LTD_4 are regulated by PKC-epsilon in a calcium-dependent manner using anti-PKC-epsilon antibodies (283). While anti-PKC-alpha and delta antibodies had no effect on LTD_4-

induced calcium. Recently, Kondeti *et al.* identified by shRNA knockdown that LTD_4 and LTE_4-induced PKC-epsilon activation was calcium-independent, while PKC-alpha negatively regulated calcium mobilisation in LAD2 cells (284). PKC-eta has never been studied as a PKC involved in cysteinyl leukotriene signalling so both isozymes need to be more comprehensively analysed to elucidate whether the increased responses to LTE_4 is a direct result of specific PKC expression and function in LAD2 cells.

Erk activation is an important signalling mechanism that links signalling events from the cytoplasm to the nucleus and is also thought to regulate receptor functionality such as internalisation through GRK and arrestins. For example, Alblas *et al.* observed significant changes in the signalling kinetics, specifically a switch from transient to sustained Erk activation, when the GPCR for neurokinin A was modified to abrogate internalisation (285,286). Eisinger *et al*. reported that morphine's ability to induce sustained Erk activation was directly attributed to its inability to prevent opioid receptor internalisation (287), while etorphine, another opioid receptor agonist, induced transient Erk activation and strongly internalised its receptor. Morphine's ability to induce internalisation was reinstated by inhibition of the Erk pathway, while etorphines was further enhanced. As desensitisation is linked to internalisation, although not exclusively, the fact that LTE_4 can only partially desensitise $CysLT_1$ signalling while LTD_4 shows full desensitisation activity suggests that $CysLT_1$ desensitisation could be potentially explained by the ligands ability to induce sustained/transient Erk activation. Although $CysLT_1$ internalisation would be very difficult to quantify, due to the unavailability of specific $CysLT_1$ antibodies, it could be hypothesised that LTE_4-induced sustained Erk activation lowers $CysLT_1$ ability to internalise and so it can only partially desensitise its receptor. LTD_4 on the other hand induces transient Erk activation that can robustly promote receptor internalisation and that is why full desensitisation can be observed. Partial desensitisation may also account for LTE_4's need for longer exposure times to be as potent as LTD_4 at inducing gene expression (Figure 6.9C).

Chapter 7

Final Discussion

These studies highlight the complexities in leukotriene receptor biology and leukotriene signalling pathways involved in the immune response in chronic diseases such as asthma. Ever since the elucidation and cloning of the two human cysteinyl leukotriene receptors, $CysLT_1$ and $CysLT_2$, LTE_4 has become the "forgotten" mediator in cysteinyl leukotriene biology (288). Its apparent weak efficacy in recombinant systems, poor binding affinities compared to LTC_4/LTD_4 and the availability of selective $CysLT_1$ antagonists side-lined LTE_4 as an important target for basic and clinical research. However, even though LTE_4 has been perceived as a weak agonist, it possesses some unique characteristics that cannot be explained by our current knowledge of the known cysteinyl leukotriene receptors. Recent studies using mouse models have brought LTE_4 back into the limelight by revealing specific LTE_4-induced inflammation that was not associated with $CysLT_1$ or $CysLT_2$ and in turn potentially identifying two GPCRs that could be responsible for such responses (170,218,236). This led us to investigate those receptors and to search for other GPCRs potentially responsible for the potent responses to LTE_4 that are observed within human cell models.

Our recombinant overexpression models as well as primary cell models analysing leukotriene mediated responses have provided strong evidence that $P2Y_{12}$ and OXGR1 are not directly activated by LTE_4, a conclusion which has been further strengthened by a recent report analysing both these receptors in human eosinophils (Chapter 3) (289). The identification of two human mast cell lines that differentially respond to LTE_4 has allowed comparative analysis to elucidate firstly the receptor involved in LTE_4 signalling and secondly the cell specific elements (signalling proteins) necessary for potent LTE_4 activity. Our data supports a new model of LTE_4 signalling in LAD2 cells, presented in Figure 7.1. It could be hypothesised that the regulation of gene expression by LTE_4 requires cells to express high levels of $G\alpha_q$ coupled $CysLT_1$. Upon stimulation this activates robust intracellular calcium mobilisation and sustained Erk activation, combined with activation of the PKC pathway. All of these elements required for LTE_4 activities are present in LAD2 cells but only partially present or absent in the case of LUVA cells.

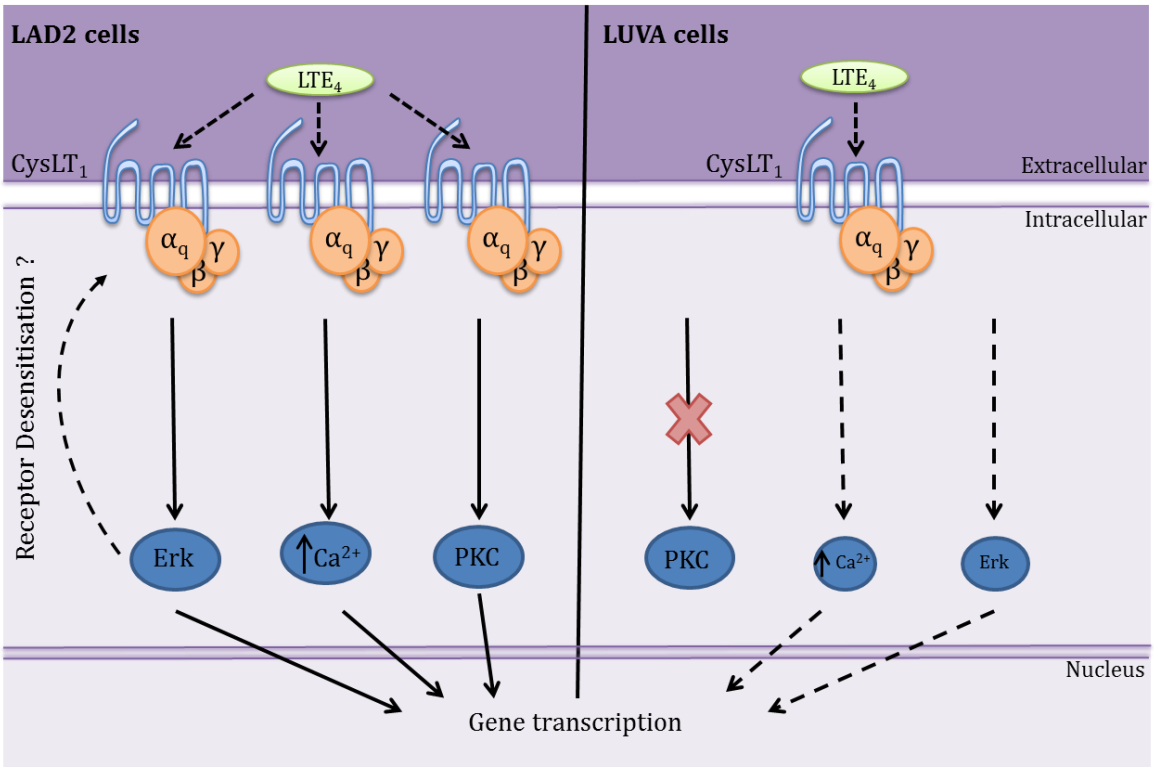

Figure 7.1 A Schematic diagram showing LTE_4 signalling pathways through $CysLT_1$ in both LAD2 and LUVA cells.

The activation of $CysLT_1$ by LTE_4 in LAD2 cells was confirmed by knockdown and selective $CysLT_1$ antagonist experiments, showing almost complete abrogation of both calcium mobilisation and gene expression (Figure 4.6 and 4.8-9). $CysLT_1$ signals via $G\alpha_q$ in both cell lines as LTE_4-induced responses were insensitive to pertussis toxin treatment in both LAD2 and LUVA cells (Figure 6.3 and 6.4). Finally, sequencing of the promoter and coding region of CYSLTR1 revealed no differences between LAD2 and LUVA cells suggesting that no CYSLTR1 genetic variations are responsible for such a selective LTE_4 activity (Chapter 5).

LTE_4 acting through $CysLT_1$ activates intracellular calcium fluxes and Erk kinases to regulate gene expression. In LAD2 but not in LUVA cells, LTE_4-induced PKC activation is required for gene transcription (Figure 6.8B). Pan-PKC inhibition partially abrogated gene expression but had no effect on calcium mobilisation suggesting that PKC activation and calcium mobilisation are regulated separately (Figure 6.8C). LTE_4-induced calcium mobilisation in both, LAD2 and LUVA cells (Figure 4.3), but a much more robust response was observed in LAD2 cells. The ability of LTE_4 to induce alternative signalling pathways in LAD2 cells, such as PKC activation and robust calcium fluxes may be a direct consequence of receptor expression levels. Analysis of mRNA levels by microarray and qRT-PCR, suggests that $CysLT_1$ is much more highly expressed in LAD2 cells than in LUVA

(Figure 6.1A and 6.2A). Certainly, in the case of LTD_4, the lower expression level that is observed in LUVA cells is sufficient to induce gene expression but for a partial agonist such as LTE_4, the constitutive receptor expression level needs to be higher to fully activate the pathway. The higher $CysLT_1$ expression in LAD2 cells would allow LTE_4 to generate a greater flux in intracellular calcium that could reach a threshold level required for regulation of gene transcription. Receptor expression is also important for the activation of multiple signalling pathways. In recombinant overexpression models, lower GPCR expression can limit signal pathway activation, a phenomenon that could explain lack of PKC pathway activation in LUVA cells (290). Similarly, PKC activation may also be influenced by constitutive expression of various PKC isozymes. Comparison of microarray data from LAD2 and LUVA cells revealed substantial differences in PKC alpha, beta, epsilon and eta expression (Figure 6.8E). PKC epsilon and eta can be activated independently of calcium and so may also contribute to the lack of PKC activation seen in LUVA cells. The involvement of the PKC pathway in cysteinyl leukotriene-induced $CysLT_1$ signal transduction has already been noted for LTD_4 (133,291). In a recent study by Kondeti *et al.* it was shown that knockdown of PKC-epsilon by siRNA in LAD2 cells can significantly inhibit LTD_4 and LTE_4-induced CCL4 release and expression of the transcription factor, c-fos (284). Although only a partial inhibition was observed, this study strengthens the idea that PKC activation is important for "normal" cysteinyl leukotriene functionality and potentially the lack of PKC expression, especially PKC-epsilon, may be critical to LTE_4 being able to achieve its "full" agonist characteristics.

Another potential factor contributing to the potency of LTE_4 is agonist exposure time. Experiments analysing long (2 hours) versus short (5 minutes) exposure times revealed that LTE_4 matched the potency of LTD_4 only with the longer exposure (Figure 6.9C). This could be a direct consequence of the inability of LTE_4 to fully desensitise $CysLT_1$, as LTD_4 can (Figure 6.10). Partial desensitisation means that receptors are still partially responsive even after the first stimulation, so that even though LTE_4 is the weaker agonist for calcium mobilisation, prolonged signalling could push gene expression to a similar level as that of LTD_4. This difference in receptor desensitisation may stem from subtle changes in Erk activation, since LTE_4 induced sustained Erk activation while that induced by LTD_4 was only transient. These changes in Erk signalling kinetics have been linked to a GPCR's ability to induce signal termination events (285-287).

Early studies analysing the effects of cysteinyl leukotriene *in vivo* revealed a disproportionate augmentation in relative responses to cysteinyl leukotriene inhalation in asthmatic patients when compared to healthy individuals (211). Arm *et al.*'s observations revealed that LTC_4 and LTD_4 were 6.2 and 9.1 fold, respectively, more potent in asthmatic

patients while LTE_4 induced a 219 fold change. Although $CysLT_1$ expression was never analysed in Arm *et al.*'s study (indeed the receptor hadn't yet been identified) recent clinical studies would suggest that $CysLT_1$ is more highly expressed in asthmatics compared to healthy individuals. For example, Zhu *et al.* have shown in bronchial biopsies of asthmatic patients that there is a significant increase in $CysLT_1$ expressing cells compared to healthy individuals (confirmed at mRNA and protein level) (292). There were also increased numbers of $CysLT_1$ expressing cells in asthmatics with frequent exacerbations compared to stable asthmatics. In patients with aspirin-exacerbated respiratory disease (AERD), an increase in $CysLT_1$ expression was observed in $CD45^+$ leukocytes in comparison to aspirin-tolerant patients (293). As the number of $CD45^+$ leukocytes did not differ between the two patient subsets, this increase in $CysLT_1$ levels was directly attributed to increase of surface expression. Aspirin desensitisation was able not only to reduce the number of $CysLT_1$ expressing leukocytes but also to blunt aspirin-induced increases in urinary LTE_4 and reduce hyperresponsiveness to LTE_4 (293). This increase in $CysLT_1$ expression in AERD patients has also been observed in mucosal inflammatory leukocytes and interestingly no changes in $CysLT_2$ expression were observed (294). Zhu *et al.* have also associated $CysLT_1$ expression with bronchial inflammation in patients with COPD (295).

Analysing data from our own studies has revealed the same disproportionate augmentation in cysteinyl leukotriene-induced responses in LAD2 cells compared to LUVA, a model of relatively high $CysLT_1$ expression versus lower $CysLT_1$ expression. LTC_4 and LTD_4 were more potent at inducing intracellular calcium mobilisation (2.7 and 2.3 respectively) in LAD2 cells compared to LUVA while LTE_4 induced a 61.8 fold change (Figure 4.2, 100 µmol/L, mean). A similar phenomenon was also observed when comparing control LAD2 cells with $CysLT_1$ knockdown LAD2, another model of relatively high $CysLT_1$ expression versus lower $CysLT_1$ expression. LTC_4 and LTD_4 again showed very similar fold changes in intracellular calcium mobilisation (1.9 and 1.8 respectively) in control LAD2 cells compared to the $CysLT_1$ knockdown LAD2 while LTE_4 was able to induce a 6.4 fold change (Figure 4.8, 100 µmol/L, mean). Arm *et al*. initially suggested that this phenomenon was attributed to LTE_4 distinct signalling properties (separate LTE_4 receptor) different from that of LTC_4 and LTD_4 that had relatively similar fold changes in responses but our data have revealed that this phenomenon is observed even within the same cell type by just lowering $CysLT_1$ expression through knockdown (212). This provides strong evidence that $CysLT_1$ expression level is one of the critical factors in how responsive individual cells are to LTE_4. As this phenomenon is inducible even within the same cell system it also suggests the genetic variations (SNPs) do not play an important role in constitutive $CysLT_1$ expression level within our human mast cell models.

A higher GPCR expression level in disease states is not an uncommon phenomenon. In multiple sclerosis (MS) many chemokine receptors are elevated in MS lesions (CCR2, CCR3, CCR5, CCR7, CXCR2), demyelinating plaques (CCR1) and in specific cell subsets, leukocytes (CCR4), T cells (CCR5, CXCR3) and monocytes (CXCR3, CXCR4) (296). Although many specific functions are still being elucidated, chemokine receptors are being utilised as therapeutic targets with two promising CCR2 modulators (MK-0812 and MLN1202) already completing phase II clinical trials (296). In cancer, GPR116 expression has been linked to breast cancer cell metastasis and a poor prognosis. PAR1, a protease-activated GPCR, has increased expression in highly invasive breast carcinomas and prostate cancer (297,298). GPCRs are highly regulated proteins and any dysfunction, including receptor overexpression, could arise from either genetic or environmental changes (or both). Our data have revealed that there are no genetic variations in the CYSLTR1 gene between LAD2 and LUVA cells and as previously stated, disproportionate responsiveness to LTE_4 can be observed within the same cell type, so genetic variations do not account for higher $CysLT_1$ expression in LAD2 cells compared to LUVA. As no specific genetic variations have been identified driving the differential $CysLT_1$ expression observed in asthmatic/rhinosinusitic and healthy individuals, it is likely that there are other factors affecting $CysLT_1$ expression. One explanation may be immunoregulation (see section 1.1.4 *Cysteinyl leukotriene receptors*). $CysLT_1$ expressing cells that are tissue resident in asthmatic patients will be exposed to an extracellular environment containing many pro-inflammatory mediators. This would be a unique feature not present in healthy individuals. These pro-inflammatory mediators could be released from activated cells i.e. mast cells themselves, recruited Th2, eosinophils and structural cells, which could all contribute to the regulation and differentiation of these cells. For example, in asthmatic patients the Th2 cell related cytokines IL-4, IL-13 and IL-5 have been shown to be significantly increased at both an mRNA and protein level in the serum, BAL and within bronchial biopsies compared to healthy individuals (299-301). IL-4, IL-13 and IL-5, which can also be released from mast cells and eosinophils, have been shown to be able to prime human mast cells to release Th2 cell associated cytokines and increase cysteinyl leukotriene production both in an IgE-dependent manner (280,302-304). What is interesting about these cytokines is that even in the absence of IgE, they are able to up-regulate $CysLT_1$ expression and so their level of "priming" could be far more extensive than just mediator production/release (121,123,126). This would suggest that the responses to LTE_4 are potentiated through an increase in $CysLT_1$ expression, occurring mainly after cell activation/priming during a chronic state of disease progression. This is reflective of the fact that leukotriene pathway antagonists have been shown to have a clear clinical benefits as therapies in adults and children with chronic asthma and are routinely

used as add on therapies for asthma that cannot be completely controlled by corticosteroids (191,305,306).

The first line treatment for asthma is inhaled corticosteroids but suppression of inflammation remains incomplete. Several studies have indicated that leukotriene levels were still detectable in the urine of moderate to severe asthmatic adults and children being treated with inhaled corticosteroids alone (307,308). Studies have also shown that the addition of a leukotriene receptor antagonist, Montelukast, to inhaled corticosteroid treatment reduces cysteinyl leukotriene and LTB_4 concentrations as well as improving symptoms (309). Eosinophilic inflammation can also be observed in asthmatics even with high dose inhaled corticosteroid treatment (310). This suggests that corticosteroids alone are insufficient to completely control airway inflammation and that the cysteinyl leukotriene pathway is relatively unaffected by such treatment. This implies that there is a necessity for leukotriene pathway antagonists in the treatment of asthma but unfortunately LTRAs such as Montelukast do have varying effects from patient to patient. This could be due to several reasons. Firstly, genetic polymorphisms within the leukotriene pathway have long been thought to be one of the main reasons for high inter-patient variability in response to treatment. Several pharmacogenetic studies have identified multiple sites that could be associated with LTRA responsiveness; for example, Asano *et al.* observed that homozygotes for the C(-444) LTC_4S allele in moderate asthmatics were more responsive to Pranlukast treatment than A(-444) homozygotes (311). Lima *et al.* observed polymorphisms in multiple genes; ALOX5, LTC_4S A(-444)C, LTA_4H and MRP1 that could influence Montelukast treatment (312). Also polymorphisms within the gene that encodes the OATP2B1 transporter protein, thought to be a carrier protein for Montelukast, have also been associated with Montelukast inconsistencies in asthma treatment (313). Unfortunately, due to the complexities of analysing a heterogeneous disease and the relatively small sample size of these studies some of these associations have already been questioned (314,315).

Secondly, Montelukast, Pranlukast and Zafirlukast are selective cysteinyl leukotriene receptor antagonists i.e. they inhibit $CysLT_1$ mediated responses and not $CysLT_2$, but they are not specific antagonists. As previously mentioned, Montelukast, Pranlukast and Zafirlukast, were all developed before any cysteinyl leukotriene receptor had been identified. Several reports have observed that these antagonists can in fact inhibit other receptors/enzymes independently of $CysLT_1$. For example, purinergic receptors (P2Y) that are activated by ADP, ATP, UDP and UTP are GPCRs that can potentiate inflammatory responses. These receptors, namely $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$, are inhibited by micromolar concentrations of Montelukast, Pranlukast and Zafirlukast in primary human monocytes

and monocyte/macrophage cell lines independently of $CysLT_1$ (263,267). Micromolar concentrations (≥1 mol/L) of Montelukast have also been shown to inhibit 5-lipoxygenase, a key enzyme in leukotriene production, in primary human monocytes and mast cell lines (human and rat) (267,316). This additional attenuation of cysteinyl leukotriene and LTB_4 production could be a complementary therapeutic property to receptor antagonism and has already generated interest in the treatment of other diseases such as cystic fibrosis (317,318). Other studies have also reported that Pranlukast can inhibit NF-κB in activated monocytes, T-cells and epithelial and endothelial cell lines independently of $CysLT_1$ and also may inhibit eosinophil adhesion to vascular cell adhesion molecule-1 (VCAM-1) (265,319-321). Although current findings suggest that cysteinyl leukotriene receptor antagonists, Montelukast, Pranlukast and Zafirlukast have additional anti-inflammatory effects, it is still theoretically possible due to the extent of off-target antagonism that some pathways may be detrimental to the original treatment.

Finally, the heterogeneous nature of asthma may play a very important role. In recent years, phenotyping asthmatic patients by clinical presentation and biological features has become of great interest for tailoring treatments. Montelukast has been shown to be very effective in specific subphenotypes of asthma. For example, in exercise-induced bronchoconstriction, Montelukast has been shown to be a more effective treatment than the long acting β_2 agonist salmeterol (322,323). Several studies have also indicated that asthmatic patients with allergic rhinitis have a significant improvement in lung function and therefore asthma control when Montelukast is added to current therapies (324-327). Montelukast may also be effective in asthmatics with low airway inflammation or in non-eosinophilic phenotypes that are not necessarily associated with cysteinyl leukotrienes although more studies need to be carried out to assess its significance. In obese asthmatics (low airway inflammation) it has been observed that Montelukast was more effective at controlling asthma than inhaled corticosteroids and in the case of viral-induced bronchoconstriction (typically non-eosinophilic) and wheezing, leukotriene receptor antagonists improved disease control although further investigations need to be carried to understand these mechanisms (328,329). However, it is clear that phenotyping of asthma might reduce the heterogeneity that is seen in Montelukast treatment. This could be directly related to the heterogeneity in $CysLT_1$ expression level. For example, in aspirin-sensitive asthmatics, $CysLT_1$ expression level on $CD45^+$ leukocytes is significantly higher than in aspirin-insensitive asthmatics and healthy individuals (294). Several studies have reported that aspirin-sensitive asthmatics are more responsive to LTE_4 than asthmatics without aspirin sensitivity, which our data have suggested, could be related to the increased $CysLT_1$ expression (212,330). Urinary LTE_4 levels are elevated in aspirin-sensitive asthmatics compared to tolerant patients indicating a constitutive

overproduction in this phenotype. Taken together, overproduction and higher sensitivity to cysteinyl leukotrienes would suggest that leukotriene receptor antagonists (or general inhibition of the leukotriene pathway) could be a very effective treatment in selected subpopulations in asthma (331-333). Although studies are limited, promising clinical trials have observed significant increases in lung function and general asthma control with LTRA treatment in aspirin-sensitive asthmatics compared to inhaled corticosteroid treatment alone (204,334).

Better characterisation of asthmatic phenotypes could lead to improved treatment and by extension fewer hospitalisations and less strain on the health care system. Unfortunately urinary LTE_4 levels, or any biological fluid measurement, are not an indicator of how responsive individuals are to cysteinyl leukotrienes. Our data suggest that $CysLT_1$ expression is a critical factor and so it would be interesting to see whether $CysLT_1$ expression is directly related to patients' response to Montelukast treatment. If there is a direct relationship then $CysLT_1$ expression level could be used as a biomarker in the characterisation of asthmatic phenotypes and therefore more effective treatments could be administered based on such characterisations.

The question whether there is a third cysteinyl leukotriene receptor still remains unanswered. The mouse models of inflammation set up by Maekawa *et al.* and Paruchuri *et al.* provide strong evidence that $CysLT_1$ and $CysLT_2$ are not the only cysteinyl leukotriene receptors as their knockout did not diminish cysteinyl leukotriene mediated inflammation (170,218). Unfortunately, the two receptors identified from these studies, $P2Y_{12}$ and OXGR1, have been clearly shown in the present study not to be directly activated by cysteinyl leukotrienes (see Chapter 3) and so this leaves the question of how LTE_4-mediated inflammation remains intact in the absence of $CysLT_1$ and $CysLT_2$ unanswered (170,236,335). Our data presented here cannot explain such a phenomenon nor do they rule out the possibility of a third cysteinyl leukotriene receptor. Our study shows for the first time that the $CysLT_1$ expression as well as a functional PKC signalling pathway, are critically important for responsiveness to LTE_4 within a human cell system. This could potentially be relevant for other human cell types other than mast cells and could thus have important implications for the treatment of specific phenotypes of asthma.

Chapter 8

References

1. Clark, J. D., Lin, L. L., Kriz, R. W., Ramesha, C. S., Sultzman, L. A., Lin, A. Y., Milona, N., and Knopf, J. L. (1991) A novel arachidonic acid-selective cytosolic PLA2 contains a Ca2+-dependent translocation domain with homology to PKC and GAP. *Cell* **65**, 1043-1051
2. Burke, J. E., and Dennis, E. A. (2009) Phospholipase A(2) structure/function, mechanism, and signaling. *Journal of Lipid Research* **50**, S237-S242
3. Stevens, R. C., Cherezov, V., Katritch, V., Abagyan, R., Kuhn, P., Rosen, H., and Wuethrich, K. (2013) The GPCR Network: a large-scale collaboration to determine human GPCR structure and function. *Nature Reviews Drug Discovery* **12**, 25-34
4. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* **289**, 739-745
5. Okada, T., Sugihara, M., Bondar, A. N., Elstner, M., Entel, P., and Buss, V. (2004) The retinal conformation and its environment in rhodopsin in light of a new 2.2 angstrom crystal structure. *Journal of Molecular Biology* **342**, 571-583
6. Standfuss, J., Xie, G., Edwards, P. C., Burghammer, M., Oprian, D. D., and Schertler, G. F. X. (2007) Crystal structure of a thermally stable rhodopsin mutant. *Journal of Molecular Biology* **372**, 1179-1188
7. Standfuss, J., Edwards, P. C., D'Antona, A., Fransen, M., Xie, G., Oprian, D. D., and Schertler, G. F. X. (2011) The structural basis of agonist-induced activation in constitutively active rhodopsin. *Nature* **471**, 656-660
8. Warne, T., Serrano-Vega, M. J., Baker, J. G., Moukhametzianov, R., Edwards, P. C., Henderson, R., Leslie, A. G. W., Tate, C. G., and Schertler, G. F. X. (2008) Structure of a beta(1)-adrenergic G-protein-coupled receptor. *Nature* **454**, 486-491
9. Lebon, G., Warne, T., Edwards, P. C., Bennett, K., Langmead, C. J., Leslie, A. G. W., and Tate, C. G. (2011) Agonist-bound adenosine A(2A) receptor structures reveal common features of GPCR activation. *Nature* **474**, 521-525
10. Warne, T., Edwards, P. C., Leslie, A. G. W., and Tate, C. G. (2012) Crystal Structures of a Stabilized beta(1)-Adrenoceptor Bound to the Biased Agonists Bucindolol and Carvedilol. *Structure* **20**, 841-849
11. Christopher, J. A., Brown, J., Dore, A. S., Errey, J. C., Koglin, M., Marshall, F. H., Myszka, D. G., Rich, R. L., Tate, C. G., Tehan, B., Warne, T., and Congreve, M. (2013) Biophysical Fragment Screening of the beta(1)-Adrenergic Receptor: Identification of High Affinity Arylpiperazine Leads Using Structure-Based Drug Design. *Journal of Medicinal Chemistry* **56**, 3446-3455
12. Miller-Gallacher, J. L., Nehme, R., Warne, T., Edwards, P. C., Schertler, G. F. X., Leslie, A. G. W., and Tate, C. G. (2014) The 2.1 angstrom Resolution Structure of Cyanopindolol-Bound beta(1)-Adrenoceptor Identifies an Intramembrane Na+ Ion that Stabilises the Ligand-Free Receptor. *Plos One* **9(3)**: e92727
13. Cherezov, V., Rosenbaum, D. M., Hanson, M. A., Rasmussen, S. G. F., Thian, F. S., Kobilka, T. S., Choi, H.-J., Kuhn, P., Weis, W. I., Kobilka, B. K., and Stevens, R. C. (2007) High-resolution crystal structure of an engineered human beta(2)-adrenergic G protein-coupled receptor. *Science* **318**, 1258-1265
14. Hanson, M. A., Cherezov, V., Griffith, M. T., Roth, C. B., Jaakola, V.-P., Chien, E. Y. T., Velasquez, J., Kuhn, P., and Stevens, R. C. (2008) A specific cholesterol binding site is established by the 2.8 angstrom structure of the human beta(2)-adrenergic receptor. *Structure* **16**, 897-905
15. Rasmussen, S. G. F., DeVree, B. T., Zou, Y., Kruse, A. C., Chung, K. Y., Kobilka, T. S., Thian, F. S., Chae, P. S., Pardon, E., Calinski, D., Mathiesen, J. M., Shah, S. T. A., Lyons, J. A., Caffrey, M., Gellman, S. H., Steyaert, J., Skiniotis, G., Weis, W. I., Sunahara, R. K., and Kobilka, B. K. (2011) Crystal structure of the beta(2) adrenergic receptor-Gs protein complex. *Nature* **477**, 549-555
16. Rasmussen, S. G. F., Choi, H.-J., Fung, J. J., Pardon, E., Casarosa, P., Chae, P. S., DeVree, B. T., Rosenbaum, D. M., Thian, F. S., Kobilka, T. S., Schnapp, A., Konetzki, I.,

Sunahara, R. K., Gellman, S. H., Pautsch, A., Steyaert, J., Weis, W. I., and Kobilka, B. K. (2011) Structure of a nanobody-stabilized active state of the beta(2) adrenoceptor. *Nature* **469**, 175-180

17. Ring, A. M., Manglik, A., Kruse, A. C., Enos, M. D., Weis, W. I., Garcia, K. C., and Kobilka, B. K. (2013) Adrenaline-activated structure of beta(2)-adrenoceptor stabilized by an engineered nanobody. *Nature* **502**, 575-579
18. Weichert, D., Kruse, A. C., Manglik, A., Hiller, C., Zhang, C., Hubner, H., Kobilka, B. K., and Gmeiner, P. (2014) Covalent agonists for studying G protein-coupled receptor activation. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 10744-10748
19. Kirshenbaum, A. S., Akin, C., Wu, Y. L., Rottem, M., Goff, J. P., Beaven, M. A., Rao, V. K., and Metcalfe, D. D. (2003) Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation aggregation of Fc epsilon RI or Fc gamma RI. *Leukemia Research* **27**, 677-682
20. Dore, A. S., Robertson, N., Errey, J. C., Ng, I., Hollenstein, K., Tehan, B., Hurrell, E., Bennett, K., Congreve, M., Magnani, F., Tate, C. G., Weir, M., and Marshall, F. H. (2011) Structure of the Adenosine A(2A) Receptor in Complex with ZM241385 and the Xanthines XAC and Caffeine. *Structure* **19**, 1283-1293
21. Hino, T., Arakawa, T., Iwanari, H., Yurugi-Kobayashi, T., Ikeda-Suno, C., Nakada-Nakura, Y., Kusano-Arai, O., Weyand, S., Shimamura, T., Nomura, N., Cameron, A. D., Kobayashi, T., Hamakubo, T., Iwata, S., and Murata, T. (2012) G-protein-coupled receptor inactivation by an allosteric inverse-agonist antibody. *Nature* **482**, 237-240
22. Wu, B., Chien, E. Y. T., Mol, C. D., Fenalti, G., Liu, W., Katritch, V., Abagyan, R., Brooun, A., Wells, P., Bi, F. C., Hamel, D. J., Kuhn, P., Handel, T. M., Cherezov, V., and Stevens, R. C. (2010) Structures of the CXCR4 Chemokine GPCR with Small-Molecule and Cyclic Peptide Antagonists. *Science* **330**, 1066-1071
23. Chien, E. Y. T., Liu, W., Zhao, Q., Katritch, V., Han, G. W., Hanson, M. A., Shi, L., Newman, A. H., Javitch, J. A., Cherezov, V., and Stevens, R. C. (2010) Structure of the Human Dopamine D3 Receptor in Complex with a D2/D3 Selective Antagonist. *Science* **330**, 1091-1095
24. Shimamura, T., Shiroishi, M., Weyand, S., Tsujimoto, H., Winter, G., Katritch, V., Abagyan, R., Cherezov, V., Liu, W., Han, G. W., Kobayashi, T., Stevens, R. C., and Iwata, S. (2011) Structure of the human histamine H-1 receptor complex with doxepin. *Nature* **475**, 65-70
25. Haga, K., Kruse, A. C., Asada, H., Yurugi-Kobayashi, T., Shiroishi, M., Zhang, C., Weis, W. I., Okada, T., Kobilka, B. K., Haga, T., and Kobayashi, T. (2012) Structure of the human M2 muscarinic acetylcholine receptor bound to an antagonist. *Nature* **482**, 547-551
26. Kruse, A. C., Ring, A. M., Manglik, A., Hu, J., Hu, K., Eitel, K., Huebner, H., Pardon, E., Valant, C., Sexton, P. M., Christopoulos, A., Felder, C. C., Gmeiner, P., Steyaert, J., Weis, W. I., Garcia, K. C., Wess, J., and Kobilka, B. K. (2013) Activation and allosteric modulation of a muscarinic acetylcholine receptor. *Nature* **504**, 101-106
27. Kruse, A. C., Hu, J., Pan, A. C., Arlow, D. H., Rosenbaum, D. M., Rosemond, E., Green, H. F., Liu, T., Chae, P. S., Dror, R. O., Shaw, D. E., Weis, W. I., Wess, J., and Kobilka, B. K. (2012) Structure and dynamics of the M3 muscarinic acetylcholine receptor. *Nature* **482**, 552-556
28. Wu, H., Wacker, D., Mileni, M., Katritch, V., Han, G. W., Vardy, E., Liu, W., Thompson, A. A., Huang, X.-P., Carroll, F. I., Mascarella, S. W., Westkaemper, R. B., Mosier, P. D., Roth, B. L., Cherezov, V., and Stevens, R. C. (2012) Structure of the human kappa-opioid receptor in complex with JDTic. *Nature* **485**, 327-332
29. Manglik, A., Kruse, A. C., Kobilka, T. S., Thian, F. S., Mathiesen, J. M., Sunahara, R. K., Pardo, L., Weis, W. I., Kobilka, B. K., and Granier, S. (2012) Crystal structure of the mu-opioid receptor bound to a morphinan antagonist. *Nature* **485**, 321-326

30. Granier, S., Manglik, A., Kruse, A. C., Kobilka, T. S., Thian, F. S., Weis, W. I., and Kobilka, B. K. (2012) Structure of the delta-opioid receptor bound to naltrindole. *Nature* **485**, 400-404
31. Fenalti, G., Giguere, P. M., Katritch, V., Huang, X.-P., Thompson, A. A., Cherezov, V., Roth, B. L., and Stevens, R. C. (2014) Molecular control of delta-opioid receptor signalling. *Nature* **506**, 191-196
32. Thompson, A. A., Liu, W., Chun, E., Katritch, V., Wu, H., Vardy, E., Huang, X.-P., Trapella, C., Guerrini, R., Calo, G., Roth, B. L., Cherezov, V., and Stevens, R. C. (2012) Structure of the nociceptin/orphanin FQ receptor in complex with a peptide mimetic. *Nature* **485**, 395-399
33. Zhang, C., Srinivasan, Y., Arlow, D. H., Fung, J. J., Palmer, D., Zheng, Y., Green, H. F., Pandey, A., Dror, R. O., Shaw, D. E., Weis, W. I., Coughlin, S. R., and Kobilka, B. K. (2012) High-resolution crystal structure of human protease-activated receptor 1. *Nature* **492**, 387-392
34. White, J. F., Noinaj, N., Shibata, Y., Love, J., Kloss, B., Xu, F., Gvozdenovic-Jeremic, J., Shah, P., Shiloach, J., Tate, C. G., and Grisshammer, R. (2012) Structure of the agonist-bound neurotensin receptor. *Nature* **490**, 508-513
35. Egloff, P., Hillenbrand, M., Klenk, C., Batyuk, A., Heine, P., Balada, S., Schlinkmann, K. M., Scott, D. J., Schuetz, M., and Plueckthun, A. (2014) Structure of signaling-competent neurotensin receptor 1 obtained by directed evolution in Escherichia coli. *Proceedings of the National Academy of Sciences of the United States of America* **111**, E655-E662
36. Park, S. H., Das, B. B., Casagrande, F., Tian, Y., Nothnagel, H. J., Chu, M., Kiefer, H., Maier, K., De Angelis, A. A., Marassi, F. M., and Opella, S. J. (2012) Structure of the chemokine receptor CXCR1 in phospholipid bilayers. *Nature* **491**, 779-783
37. Hanson, M. A., Roth, C. B., Jo, E., Griffith, M. T., Scott, F. L., Reinhart, G., Desale, H., Clemons, B., Cahalan, S. M., Schuerer, S. C., Sanna, M. G., Han, G. W., Kuhn, P., Rosen, H., and Stevens, R. C. (2012) Crystal Structure of a Lipid G Protein-Coupled Receptor. *Science* **335**, 851-855
38. Siu, F. Y., He, M., de Graaf, C., Han, G. W., Yang, D., Zhang, Z., Zhou, C., Xu, Q., Wacker, D., Joseph, J. S., Liu, W., Lau, J., Cherezov, V., Katritch, V., Wang, M.-W., and Stevens, R. C. (2013) Structure of the human glucagon class B G-protein-coupled receptor. *Nature* **499**, 444-449
39. Wang, C., Wu, H., Katritch, V., Han, G. W., Huang, X.-P., Liu, W., Siu, F. Y., Roth, B. L., Cherezov, V., and Stevens, R. C. (2013) Structure of the human smoothened receptor bound to an antitumour agent. *Nature* **497**, 338-343
40. Weierstall, U., James, D., Wang, C., White, T. A., Wang, D., Liu, W., Spence, J. C. H., *et al.* (2014) Lipidic cubic phase injector facilitates membrane protein serial femtosecond crystallography. *Nature Communications* **5**:3309
41. Wang, C., Wu, H., Evron, T., Vardy, E., Han, G. W., Huang, X.-P., Hufeisen, S. J., Mangano, T. J., Urban, D. J., Katritch, V., Cherezov, V., Caron, M. G., Roth, B. L., and Stevens, R. C. (2014) Structural basis for Smoothened receptor modulation and chemoresistance to anticancer drugs. *Nature communications* **5**:4355
42. Wacker, D., Wang, C., Katritch, V., Han, G. W., Huang, X.-P., Vardy, E., McCorvy, J. D., Jiang, Y., Chu, M., Siu, F. Y., Liu, W., Xu, H. E., Cherezov, V., Roth, B. L., and Stevens, R. C. (2013) Structural Features for Functional Selectivity at Serotonin Receptors. *Science* **340**, 615-619
43. Wang, C., Jiang, Y., Ma, J., Wu, H., Wacker, D., Katritch, V., Han, G. W., Liu, W., Huang, X.-P., Vardy, E., McCorvy, J. D., Gao, X., Zhou, X. E., Melcher, K., Zhang, C., Bai, F., Yang, H., Yang, L., Jiang, H., Roth, B. L., Cherezov, V., Stevens, R. C., and Xu, H. E. (2013) Structural Basis for Molecular Recognition at Serotonin Receptors. *Science* **340**, 610-614
44. Liu, W., Wacker, D., Gati, C., Han, G. W., James, D., Wang, D., Nelson, G., *et al.* (2013) Serial Femtosecond Crystallography of G Protein-Coupled Receptors. *Science* **342**, 1521-1524

45. Tan, Q., Zhu, Y., Li, J., Chen, Z., Han, G. W., Kufareva, I., Li, T., Ma, L., Fenalti, G., Li, J., Zhang, W., Xie, X., Yang, H., Jiang, H., Cherezov, V., Liu, H., Stevens, R. C., Zhao, Q., and Wu, B. (2013) Structure of the CCR5 Chemokine Receptor-HIV Entry Inhibitor Maraviroc Complex. *Science* **341**, 1387-1390
46. Dore, A., Okrasa, K., Patel, J., Serrano-Vega, M., Bennett, K., Cooke, R., Errey, J., Jazayeri, A., Khan, S., Tehan, B., Weir, M., Wiggin, G., and Marshall, F. (2014) Structure of the human class C GPCR metabotropic glutamate receptor 5 transmembrane domain in complex with the negative allosteric modulator mavoglurant. Nature **511**, 557-562
47. Zhang, K., Zhang, J., Gao, Z.-G., Zhang, D., Zhu, L., Han, G. W., Moss, S. M., Paoletta, S., Kiselev, E., Lu, W., Fenalti, G., Zhang, W., Mueller, C. E., Yang, H., Jiang, H., Cherezov, V., Katritch, V., Jacobson, K. A., Stevens, R. C., Wu, B., and Zhao, Q. (2014) Structure of the human P2Y(12) receptor in complex with an antithrombotic drug. *Nature* **509**, 115-118
48. Zhang, J., Zhang, K., Gao, Z.-G., Paoletta, S., Zhang, D., Han, G. W., Li, T., Ma, L., Zhang, W., Mueller, C. E., Yang, H., Jiang, H., Cherezov, V., Katritch, V., Jacobson, K. A., Stevens, R. C., Wu, B., and Zhao, Q. (2014) Agonist-bound structure of the human P2Y(12) receptor. *Nature* **509**, 119-122
49. Kobilka, B. K. (2007) G protein coupled receptor structure and activation. *Biochimica Et Biophysica Acta-Biomembranes* **1768**, 794-807
50. Lagerstrom, M. C., and Schioth, H. B. (2008) Structural diversity of G protein-coupled receptors and significance for drug discovery. *Nature Reviews Drug Discovery* **7**, 339-357
51. Jingami, H., Nakanishi, S., and Morikawa, K. (2003) Structure of the metabotropic glutamate receptor. *Current Opinion in Neurobiology* **13**, 271-278
52. Henzler-Wildman, K., and Kern, D. (2007) Dynamic personalities of proteins. *Nature* **450**, 964-972
53. Nygaard, R., Zou, Y., Dror, R. O., Mildorf, T. J., Arlow, D. H., Manglik, A., Pan, A. C., Liu, C. W., Fung, J. J., Bokoch, M. P., Thian, F. S., Kobilka, T. S., Shaw, D. E., Mueller, L., Prosser, R. S., and Kobilka, B. K. (2013) The Dynamic Process of beta(2)-Adrenergic Receptor Activation. *Cell* **152**, 532-542
54. Schwartz, T. W., Frimurer, T. M., Holst, B., Rosenkilde, M. M., and Elling, C. E. (2006) Molecular mechanism of 7TM receptor activation - A global toggle switch model. *Annual Review of Pharmacology and Toxicology* **46**, 481-519
55. Scheerer, P., Park, J. H., Hildebrand, P. W., Kim, Y. J., Krauss, N., Choe, H.-W., Hofmann, K. P., and Ernst, O. P. (2008) Crystal structure of opsin in its G-protein-interacting conformation. *Nature* **455**, 497-502
56. Odowd, B. F., Hnatowich, M., Regan, J. W., Leader, W. M., Caron, M. G., and Lefkowitz, R. J. (1988) Site-directed mutagenesis of the cytoplasmic domains of the human beta-2-adrenergic receptor-localization of regions involved in G-protein-receptor coupling. *Journal of Biological Chemistry* **263**, 15985-15992
57. Oldham, W. M., and Hamm, H. E. (2008) Heterotrimeric G protein activation by G-protein-coupled receptors. *Nature Reviews Molecular Cell Biology* **9**, 60-71
58. Downes, G. B., and Gautam, N. (1999) The G protein subunit gene families. *Genomics* **62**, 544-552
59. Mizuno, N., and Itoh, H. (2009) Functions and Regulatory Mechanisms of Gq-Signaling Pathways. *Neurosignals* **17**, 42-54
60. Mischak, H., Goodnight, J., Kolch, W., Martinybaron, G., Schaechtle, C., Kazanietz, M. G., Blumberg, P. M., Pierce, J. H., and Mushinski, J. F. (1993) Overexpression of protein kinase-C-delta and kinase-epsilon in NIH 3T3 cells induces opposite effects on growth, morphology, anchorage dependence and tumorigenicity. *Journal of Biological Chemistry* **268**, 6090-6096
61. Mangmool, S., and Kurose, H. (2011) G(i/o) Protein-Dependent and -Independent Actions of Pertussis Toxin (PTX). *Toxins* **3**, 884-899

62. Zemlickova, E., Johannes, F. J., Aitken, A., and Dubois, T. (2004) Association of CPI-17 with protein kinase C and casein kinase I. *Biochemical and Biophysical Research Communications* **316**, 39-47
63. Goode, N., Hughes, K., Woodgett, J. R., and Parker, P. J. (1992) Differential regulation of glycogen-synthase kinase-3-beta by protein-kinase-C-isotypes. *Journal of Biological Chemistry* **267**, 16878-16882
64. Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marme, D., and Rapp, U. R. (1993) Protein kinase -C-alpha activates Raf-1 by direct phosphorylation. *Nature* **364**, 249-252
65. Berridge, M. J. (1993) Inositol triphosphate and calcium signalling. *Nature* **361**, 315-325
66. Chan, J., Whitten, A. E., Jeffries, C. M., Bosanac, I., Mal, T. K., Ito, J., Porumb, H., Michikawa, T., Mikoshiba, K., Trewhella, J., and Ikura, M. (2007) Ligand-induced conformational changes via flexible linkers in the amino-terminal region of the inositol 1,4,5-trisphosphate receptor. *Journal of Molecular Biology* **373**, 1269-1280
67. Purves, G. I., Kamishima, T., Davies, L. M., Quayle, J. M., and Dart, C. (2009) Exchange protein activated by cAMP (Epac) mediates cAMP-dependent but protein kinase A-insensitive modulation of vascular ATP-sensitive potassium channels. *Journal of Physiology-London* **587**, 3639-3650
68. Delghandi, M. P., Johannessen, M., and Moens, U. (2005) The cAMP signalling pathway activates CREB through PKA, p38 and MSK1 in NIH 3T3 cells. *Cellular Signalling* **17**, 1343-1351
69. Plyte, S. E., Hughes, K., Nikolakaki, E., Pulverer, B. J., and Woodgett, J. R. (1992) Glycogen-synthase kinase-3 - Functions in oncogenesis and development. *Biochimica Et Biophysica Acta* **1114**, 147-162
70. Luttrell, D. K., and Luttrell, L. M. (2004) Not so strange bedfellows: G-protein-coupled receptors and Src family kinases. *Oncogene* **23**, 7969-7978
71. Antonelli, V., Bernasconi, F., Wong, Y. H., and Vallar, L. (2000) Activation of B-Raf and regulation of the mitogen-activated protein kinase pathway by the G(o) alpha chain. *Molecular Biology of the Cell* **11**, 1129-1142
72. Sit, S.-T., and Manser, E. (2011) Rho GTPases and their role in organizing the actin cytoskeleton. *Journal of Cell Science* **124**, 679-683
73. Ui, M. (1984) Islet-activating protein, pertussis toxin- A probe for functions of the inhibitory guanine-nucleotide regulatory component of adenylate-cyclase. *Trends in Pharmacological Sciences* **5**, 277-279
74. Drew, B. A., Burow, M. E., and Beckman, B. S. (2012) MEK5/ERK5 pathway: The first fifteen years. *Biochimica Et Biophysica Acta-Reviews on Cancer* **1825**, 37-48
75. Buhl, A. M., Johnson, N. L., Dhanasekaran, N., and Johnson, G. L. (1995) G-alpha(12) and G-alpha(13) stimulate Rho-dependent stress fiber formation and focal adhesion assembly. *Journal of Biological Chemistry* **270**, 24631-24634
76. Fromm, C., Coso, O. A., Montaner, S., Xu, N. Z., and Gutkind, J. S. (1997) The small GTP-binding protein Rho links G protein-coupled receptors and G alpha(12) to the serum response element and to cellular transformation. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 10098-10103
77. Luttrell, L. M., Ferguson, S. S. G., Daaka, Y., Miller, W. E., Maudsley, S., Della Rocca, G. J., Lin, F. T., Kawakatsu, H., Owada, K., Luttrell, D. K., Caron, M. G., and Lefkowitz, R. J. (1999) beta-arrestin-dependent formation of beta(2) adrenergic receptor Src protein kinase complexes. *Science* **283**, 655-661
78. DeFea, K. A., Zalevsky, J., Thoma, M. S., Dery, O., Mullins, R. D., and Bunnett, N. W. (2000) beta-Arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. *Journal of Cell Biology* **148**, 1267-1281
79. Barlic, J., Andrews, J. D., Kelvin, A. A., Bosinger, S. E., DeVries, M. E., Xu, L. L., Dobransky, T., Feldman, R. D., Ferguson, S. S. G., and Kelvin, D. J. (2000) Regulation

of tyrosine kinase activation and granule release through beta-arrestin by CXCRI. *Nature Immunology* **1**, 227-233

80. Luttrell, L. M., Roudabush, F. L., Choy, E. W., Miller, W. E., Field, M. E., Pierce, K. L., and Lefkowitz, R. J. (2001) Activation and targeting of extracellular signal-regulated kinases by beta-arrestin scaffolds. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 2449-2454
81. Guo, C., and Whitmarsh, A. J. (2008) The beta-arrestin-2 scaffold protein promotes c-jun N-terminal kinase-3 activation by binding to its nonconserved N terminus. *Journal of Biological Chemistry* **283**, 15903-15911
82. Schwartz, T. W. (1994) Locating ligand-binding sites in 7TM receptors by protein engineering. *Current Opinion in Biotechnology* **5**, 434-444
83. Feldberg, W., Holden, H. F., and Kellaway, C. H. (1938) The formation of lysocithin and of a muscle-stimulating substance by snake venoms. *Journal of Physiology-London* **94**, 232-248
84. Kellaway, C. H., and Trethewie, E. R. (1940) The liberation of a slow-reacting smooth muscle-stimulating substance in anaphylaxis. *Quarterly Journal of Experimental Physiology and Cognate Medical Sciences* **30**, 121-145
85. Brocklehurst, W. E. (1960) The release of histamine and formation of a slow-reacting substance (SRS-A) during anaphylactic shock. *Journal of Physiology-London* **151**, 416-435
86. Brocklehurst, W. E. (1953) Occurrence of an unidentified substaance during anaphylactic shock in cavy lung. *Journal of Physiology-London* **120**, P16-P17
87. Orange, R. P., Murphy, R. C., Karnovsk.Ml, and Austen, K. F. (1973) Physicochemical characteristics and purification of slow-reacting substance of anaphylaxis. *Journal of Immunology* **110**, 760-770
88. Strandbe.K, and Uvnas, B. (1971) Purification and properties of slow reacting substance formed in cat paw perfused with compound 48/80. *Acta Physiologica Scandinavica* **82**, 358-&
89. Jakschik, B. A., Falkenhein, S., and Parker, C. W. (1977) Precursor role of archidonic-acid in release of slow reacting substance from rat basophilic leukemia-cells. *Proceedings of the National Academy of Sciences of the United States of America* **74**, 4577-4581
90. Bach, M. K., Brashler, J. R., and Gorman, R. R. (1977) Structure of slow reacting substance of anaphylaxis- Evidence of biosynthesis from arachidonic acid. *Prostaglandins* **14**, 21-38
91. Hammarstrom, S., Murphy, R. C., Samuelsson, B., Clark, D. A., Mioskowski, C., and Corey, E. J. (1979) Structure of leukotriene-C identification of the amino-acid part. *Biochemical and Biophysical Research Communications* **91**, 1266-1272
92. Murphy, R. C., Hammarstrom, S., and Samuelsson, B. (1979) Leukotriene-C- Slow-reacting substance from murine mastocytoma-cells. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 4275-4279
93. Rouzer, C. A., and Kargman, S. (1988) Translocation of 5-lipoxygenase to the membrane in human-leukocytes challenged with ionophore-A23187. *Journal of Biological Chemistry* **263**, 10980-10988
94. Mandal, A. K., Jones, P. B., Bair, A. M., Christmas, P., Miller, D., Yamin, T.-t. D., Wisniewski, D., Menke, J., Evans, J. F., Hyman, B. T., Bacskai, B., Chen, M., Lee, D. M., Nikolic, B., and Soberman, R. J. (2008) The nuclear membrane organization of leukotriene synthesis. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 20434-20439
95. Peters-Golden, M., and Brock, T. G. (2001) Intracellular compartmentalization of leukotriene synthesis: unexpected nuclear secrets. *Febs Letters* **487**, 323-326
96. Martinez Molina, D., Wetterholm, A., Kohl, A., McCarthy, A. A., Niegowski, D., Ohlson, E., Hammarberg, T., Eshaghi, S., Haeggstrom, J. Z., and Nordlund, P. (2007) Structural basis for synthesis of inflammatory mediators by human leukotriene C4 synthase. *Nature* **448**, 613-616

97. Ding, G. Y., Shen, T. J., and Center, M. S. (1999) Multidrug resistance-associated protein (MRP) mediated transport of daunomycin and leukotriene C4 (LTC4) in isolated plasma membrane vesicles. *Anticancer Research* **19**, 3243-3248
98. Loe, D. W., Almquist, K. C., Deeley, R. G., and Cole, S. P. C. (1996) Multidrug resistance protein (MRP)-mediated transport of leukotriene C-4 and chemotherapeutic agents in membrane vesicles - Demonstration of glutathione-dependent vincristine transport. *Journal of Biological Chemistry* **271**, 9675-9682
99. Rius, M., Hummel-Eisenbeiss, J., and Keppler, D. (2008) ATP-dependent transport of Leukotrienes B-4 and C-4 by the multidrug resistance protein ABCC4 (MRP4). *Journal of Pharmacology and Experimental Therapeutics* **324**, 86-94
100. Zarini, S., Gijon, M. A., Ransome, A. E., Murphy, R. C., and Sala, A. (2009) Transcellular biosynthesis of cysteinyl leukotrienes in vivo during mouse peritoneal inflammation. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 8296-8301
101. Folco, G., and Murphy, R. C. (2006) Eicosanoid transcellular biosynthesis: From cell-cell interactions to in vivo tissue responses. *Pharmacological Reviews* **58**, 375-388
102. Orning, L., and Hammarstrom, S. (1982) Kinetics of the conversion of leukotriene-C by gamma-glutamyl-transferase transpeptidase. *Biochemical and Biophysical Research Communications* **106**, 1304-1309
103. Bernstrom, K., Orning, L., and Hammarstrom, S. (1982) Gamma-glutamyl-transferase transpeptidase, a leukotriene metabolizing enzyme. *Methods in Enzymology* **86**, 38-45
104. Anderson, M. E., Allison, R. D., and Meister, A. (1982) Interconversion of leukotrienes catalyzed by purified gamma-glutamyl-transferase transpeptidase-concomitant formation of leukotriene D4 and gamma-glutamyl-transferase amino-acids. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences* **79**, 1088-1091
105. Lee, C. W., Lewis, R. A., Corey, E. J., and Austen, K. F. (1983) Conversion of leukotriene-D4 to Leukotriene-E4 by a dipeptidase released from the specific granule of human polymorphonuclear leukocytes. *Immunology* **48**, 27-35
106. Jones, T. R., Labelle, M., Belley, M., Champion, E., Charette, L., Evans, J., Fordhutchinson, A. W., Gauthier, J. Y., Lord, A., Masson, P., McAuliffe, M., McFarlane, C. S., Metters, K. M., Pickett, C., Piechuta, H., Rochette, C., Rodger, I. W., Sawyer, N., Young, R. N., Zamboni, R., and Abraham, W. M. (1995) Pharmacology of Montelukast sodium (Singulair™), a potent and selective leukotriene D-4 receptor antagonist. *Canadian Journal of Physiology and Pharmacology* **73**, 191-201
107. Obata, T., Nambu, F., Kitagawa, T., Terashima, H., Toda, M., Okegawa, T., and Kawasaki, A. (1987) ONO-1078: an antagonist of leukotrienes. *Advances in prostaglandin, thromboxane, and leukotriene research* **17A**, 540-543
108. Krell, R. D., Aharony, D., Buckner, C. K., Keith, R. A., Kusner, E. J., Snyder, D. W., Bernstein, P. R., Matassa, V. G., Yee, Y. K., Brown, F. J., Hesp, B., and Giles, R. E. (1990) The preclinical pharmacology of ICI-204,219 - a peptide leukotriene antagonist. *American Review of Respiratory Disease* **141**, 978-987
109. Ghodbane, S., and McCauley, J. A. (1990) Study of the polymorphism of 3-(((3-(2-(7-chloro-2-quinolinyl)(E)-ethenyl)phenyl)((3-(dimethylamino-3-oxopropyl)thio)methyl)thio)propanoic acid(MK571) by DSC, TG, XRPD and solubility measurements. *International Journal of Pharmaceutics* **59**, 281-286
110. Tudhope, S. R., Cuthbert, N. J., Abram, T. S., Jennings, M. A., Maxey, R. J., Thompson, A. M., Norman, P., and Gardiner, P. J. (1994) BAY-U9773, A novel antagonist of cyteinyl -leukotrienes with activity against 2 receptor subtypes. *European Journal of Pharmacology* **264**, 317-323
111. Capra, V., Nicosia, S., Ragnini, D., Mezzetti, M., Keppler, D., and Rovati, G. E. (1998) Identification and characterization of two cysteinyl-leukotriene high affinity

binding sites with receptor characteristics in human lung parenchyma. *Molecular Pharmacology* **53**, 750-758

112. Lewis, M. A., Mong, S., Vessella, R. L., and Crooke, S. T. (1985) Identification and characterization of leukotriene D4 receptors in adult and fetal human-lung. *Biochemical Pharmacology* **34**, 4311-4317
113. Lynch, K. R., O'Neill, G. P., Liu, Q. Y., Im, D. S., Sawyer, N., Metters, K. M., Coulombe, N., Abramovitz, M., Figueroa, D. J., Zeng, Z. Z., Connolly, B. M., Bai, C., Austin, C. P., Chateauneuf, A., Stocco, R., Greig, G. M., Kargman, S., Hooks, S. B., Hosfield, E., Williams, D. L., Ford-Hutchinson, A. W., Caskey, C. T., and Evans, J. F. (1999) Characterization of the human cysteinyl leukotriene CysLT(1) receptor. *Nature* **399**, 789-793
114. Sarau, H. M., Ames, R. S., Chambers, J., Ellis, C., Elshourbagy, N., Foley, J. J., Schmidt, D. B., Muccitelli, R. M., Jenkins, O., Murdock, P. R., Herrity, N. C., Halsey, W., Sathe, G., Muir, A. I., Nuthulaganti, P., Dytko, G. M., Buckley, P. T., Wilson, S., Bergsma, D. J., and Hay, D. W. P. (1999) Identification, molecular cloning, expression, and characterization of a cysteinyl leukotriene receptor. *Molecular Pharmacology* **56**, 657-663
115. Heise, C. E., O'Dowd, B. F., Figueroa, D. J., Sawyer, N., Nguyen, T., Im, D. S., Stocco, R., Bellefeuille, J. N., Abramovitz, M., Cheng, R., Williams, D. L., Zeng, Z. Z., Liu, Q. Y., Ma, L., Clements, M. K., Coulombe, N., Liu, Y., Austin, C. P., George, S. R., O'Neill, G. P., Metters, K. M., Lynch, K. R., and Evans, J. F. (2000) Characterization of the human cysteinyl leukotriene 2 receptor. *Journal of Biological Chemistry* **275**, 30531-30536
116. Nothacker, H. P., Wang, Z. W., Zhu, Y. H., Reinscheid, R. K., Lin, S. H. S., and Civelli, O. (2000) Molecular cloning and characterization of a second human cysteinyl leukotriene receptor: Discovery of a subtype selective agonist. *Molecular Pharmacology* **58**, 1601-1608
117. Takasaki, J., Kamohara, M., Matsumoto, M., Saito, T., Sugimoto, T., Ohishi, T., Ishii, H., Ota, T., Nishikawa, T., Kawai, Y., Masuho, Y., Isogai, T., Suzuki, Y., Sugano, S., and Furuichi, K. (2000) The molecular characterization and tissue distribution of the human cysteinyl leukotriene CysLT(2) receptor. *Biochemical and Biophysical Research Communications* **274**, 316-322
118. Woszczek, G., Pawliczak, R., Qi, H. Y., Nagineni, S., Alsaaty, S., Logun, C., and Shelhamer, J. H. (2005) Functional characterization of human cysteinyl leukotriene 1 receptor gene structure. *Journal of Immunology* **175**, 5152-5159
119. Sharman, J. L., Benson, H. E., Pawson, A. J., Lukito, V., Mpamhanga, C. P., Bombail, V., Davenport, A. P., Peters, J. A., Spedding, M., Harmar, A. J., and Nc, I. (2013) IUPHAR-DB: updated database content and new features. *Nucleic Acids Research* **41**, D1083-D1088
120. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J. H., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**, 3389-3402
121. Thivierge, M., Doty, M., Johnson, J., Stankova, J., and Rola-Pleszczynski, M. (2000) IL-5 up-regulates cysteinyl leukotriene 1 receptor expression in HL-60 cells differentiated into eosinophils. *Journal of Immunology* **165**, 5221-5226
122. Figueroa, D. J., Breyer, R. M., Defoe, S. K., Kargman, S., Daugherty, B. L., Waldburger, K., Liu, Q. Y., Clements, M., Zeng, Z. Z., O'Neill, G. P., Jones, T. R., Lynch, K. R., Austin, C. P., and Evans, J. F. (2001) Expression of the cysteinyl leukotriene 1 receptor in normal human lung and peripheral blood leukocytes. *American Journal of Respiratory and Critical Care Medicine* **163**, 226-233
123. Thivierge, M., Stankova, J., and Rola-Pleszczynski, M. (2001) IL-13 and IL-4 up-regulate cysteinyl leukotriene 1 receptor expression in human monocytes and macrophages. *Journal of Immunology* **167**, 2855-2860
124. Thivierge, M., Stankova, J., and Rola-Pleszczynski, M. (2006) Toll-like receptor agonists differentially regulate cysteinyl-leukotriene receptor 1 expression and

function in human dendritic cells. *Journal of Allergy and Clinical Immunology* **117**, 1155-1162

125. Mellor, E. A., Maekawa, A., Austen, K. F., and Boyce, J. A. (2001) Cysteinyl leukotriene receptor 1 is also a pyrimidinergic receptor and is expressed by human mast cells. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 7964-7969
126. Chibana, K., Ishii, Y., Asakura, T., and Fukuda, T. (2003) Up-regulation of cysteinyl leukotriene 1 receptor by IL-13 enables human lung fibroblasts to respond to leukotriene C-4 and produce eotaxin. *Journal of Immunology* **170**, 4290-4295
127. Huang, X.-Q., Zhang, X.-Y., Wang, X.-R., Yu, S.-Y., Fang, S.-H., Lu, Y.-B., Zhang, W.-P., and Wei, E.-Q. (2012) Transforming growth factor beta 1-induced astrocyte migration is mediated in part by activating 5-lipoxygenase and cysteinyl leukotriene receptor 1. *Journal of Neuroinflammation* **9**:145
128. Eaton, A., Nagy, E., Pacault, M., Fauconnier, J., and Back, M. (2012) Cysteinyl leukotriene signaling through perinuclear CysLT(1) receptors on vascular smooth muscle cells transduces nuclear calcium signaling and alterations of gene expression. *Journal of Molecular Medicine-Jmm* **90**, 1223-1231
129. Parhamifar, L., Sime, W., Yudina, Y., Vilhardt, F., Morgelin, M., and Sjolander, A. (2010) Ligand-Induced Tyrosine Phosphorylation of Cysteinyl Leukotriene Receptor 1 Triggers Internalization and Signaling in Intestinal Epithelial Cells. *Plos One* **5(12)**:e14439
130. Parmentier, C. N., Fuerst, E., McDonald, J., Bowen, H., Lee, T. H., Pease, J. E., Woszczek, G., and Cousins, D. J. (2012) Human T(H)2 cells respond to cysteinyl leukotrienes through selective expression of cysteinyl leukotriene receptor 1. *Journal of Allergy and Clinical Immunology* **129**, 1136-1142
131. Doherty, T. A., Khorram, N., Lund, S., Mehta, A. K., Croft, M., and Broide, D. H. (2013) Lung type 2 innate lymphoid cells express cysteinyl leukotriene receptor 1, which regulates T(H)2 cytokine production. *Journal of Allergy and Clinical Immunology* **132**, 205-213
132. Labat, C., Ortiz, J. L., Norel, X., Gorenne, I., Verley, J., Abram, T. S., Cuthbert, N. J., Tudhope, S. R., Norman, P., Gardiner, P., and Brink, C. (1992) A 2nd cysteinyl leukotriene receptor in human lung. *Journal of Pharmacology and Experimental Therapeutics* **263**, 800-805
133. Hoshino, M., Izumi, T., and Shimizu, T. (1998) Leukotriene D-4 activates mitogen-activated protein kinase through a protein kinase C alpha Raf-1-dependent pathway in human monocytic leukemia THP-1 cells. *Journal of Biological Chemistry* **273**, 4878-4882
134. Woszczek, G., Chen, L.-Y., Nagineni, S., Kern, S., Barb, J., Munson, P. J., Logun, C., Danner, R. L., and Shelhamer, J. H. (2008) Leukotriene D-4 induces gene expression in human monocytes through cysteinyl leukotriene type I receptor. *Journal of Allergy and Clinical Immunology* **121**, 215-221
135. Woszczek, G., Chen, L.-Y., Nagineni, S., Alsaaty, S., Harry, A., Logun, C., Pawliczak, R., and Shelhamer, J. H. (2007) IFN-gamma induces cysteinyl leukotriene receptor 2 expression and enhances the responsiveness of human endothelial cells to cysteinyl leukotrienes. *Journal of Immunology* **178**, 5262-5270
136. Zhang, W. P., Hu, H., Zhang, L., Ding, W., Yao, H. T., Chen, K. D., Sheng, W. W., Chen, Z., and Wei, E. Q. (2004) Expression of cysteinyl leukotriene receptor 1 in human traumatic brain injury and brain tumors. *Neuroscience Letters* **363**, 247-251
137. Wunder, F., Tinel, H., Kast, R., Geerts, A., Becker, E. M., Kolkhof, P., Huetter, J., Ergueden, J., and Haerter, M. (2010) Pharmacological characterization of the first potent and selective antagonist at the cysteinyl leukotriene 2 (CysLT(2)) receptor. *British Journal of Pharmacology* **160**, 399-409
138. Kamohara, M., Takasaki, J., Matsumoto, M., Matsumoto, S., Saito, T., Soga, T., Matsushime, H., and Furuichi, K. (2001) Functional characterization of cysteinyl

leukotriene CysLT(2) receptor on human coronary artery smooth muscle cells. *Biochemical and Biophysical Research Communications* **287**, 1088-1092

139. Carnini, C., Accomazzo, M. R., Borroni, E., Vitellaro-Zuccarello, L., Durand, T., Folco, G., Rovati, G. E., Capra, V., and Sala, A. (2011) Synthesis of cysteinyl leukotrienes in human endothelial cells: subcellular localization and autocrine signaling through the CysLT(2) receptor. *Faseb Journal* **25**, 3519-3528
140. Mellor, E. A., Frank, N., Soler, D., Hodge, M. R., Lora, J. M., Austen, K. F., and Boyce, J. A. (2003) Expression of the type 2 receptor for cysteinyl leukotrienes (CysLT2R) by human mast cells: Functional distinction from CysLT1R. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 11589-11593
141. Thompson, C., Cloutier, A., Bosse, Y., Poisson, C., Larivee, P., McDonald, P. P., Stankova, J., and Rola-Pleszczynski, M. (2008) Signaling by the cysteinyl-leukotriene receptor 2 - Involvement in chemokine gene transcription. *Journal of Biological Chemistry* **283**, 1974-1984
142. Wieland, G. D., Nehmann, N., Muller, D., Eibel, H., Siebenlist, U., Suhnel, J., Zipfel, P. F., and Skerka, C. (2005) Early growth response proteins EGR-4 and EGR-3 interact with immune inflammatory mediators NF-kappa B p50 and p65. *Journal of Cell Science* **118**, 3203-3212
143. Early, S. B., Barekzi, E., Negri, J., Hise, K., Borish, L., and Steinke, J. W. (2007) Concordant modulation of cysteinyl leukotriene receptor expression by IL-4 and IFN-gamma on peripheral immune cells. *American Journal of Respiratory Cell and Molecular Biology* **36**, 715-720
144. Shirasaki, H., Seki, N., Fujita, M., Kikuchi, M., Kanaizumi, E., Watanabe, K., and Himi, T. (2007) Agonist- and T(H)2 cytokine-induced upregulation of cysteinyl leukotriene receptor messenger RNA in human monocytes. *Annals of Allergy Asthma & Immunology* **99**, 340-347
145. Mellor, E. A., Austen, K. F., and Boyce, J. A. (2002) Cysteinyl leukotrienes and uridine diphosphate induce cytokine generation by human mast cells through an interleukin 4-regulated pathway that is inhibited by leukotriene receptor antagonists. *Journal of Experimental Medicine* **195**, 583-592
146. Takeda, K., Tanaka, T., Shi, W., Matsumoto, M., Minami, M., Kashiwamura, S., Nakanishi, K., Yoshida, N., Kishimoto, T., and Akira, S. (1996) Essential role of Stat6 in IL-4 signalling. *Nature* **380**, 627-630
147. Kaplan, M. H., Schindler, U., Smiley, S. T., and Grusby, M. J. (1996) Stat6 is required for mediating responses to IL-4 and for the development of Th2 cells. *Immunity* **4**, 313-319
148. Lamoureux, J., Stankova, J., and Rola-Pleszczynski, M. (2006) Leukotriene D-4 enhances immunoglobulin production in CD40-activated human B lymphocytes. *Journal of Allergy and Clinical Immunology* **117**, 924-930
149. Espinosa, K., Bosse, Y., Stankova, J., and Rola-Pleszczynski, M. (2003) CysLT1 receptor upregulation by TGF-beta and IL-13 is associated with bronchial smooth muscle cell proliferation in response to LTD4. *Journal of Allergy and Clinical Immunology* **111**, 1032-1040
150. Amrani, Y., Moore, P. E., Hoffman, R., Shore, S. A., and Panettieri, R. A. (2001) Interferon-gamma modulates cysteinyl leukotriene receptor-1 expression and function in human airway myocytes. *American Journal of Respiratory and Critical Care Medicine* **164**, 2098-2101
151. Fujii, M., Tanaka, H., and Abe, S. (2005) Interferon-gamma up-regulates expression of cysteinyl leukotriene type 2 receptors on eosinophils in asthmatic patients. *Chest* **128**, 3148-3155
152. Sjostrom, M., Johansson, A. S., Schroder, O., Qiu, H., Palmblad, J., and Haeggstrom, J. Z. (2003) Dominant expression of the CysLT(2) receptor accounts for calcium signaling by cysteinyl leukotrienes in human umbilical vein endothelial cells. *Arteriosclerosis Thrombosis and Vascular Biology* **23**, E37-E41

153. Lotzer, K., Spanbroek, R., Hildner, M., Urbach, A., Heller, R., Bretschnedier, E., Galczenski, H., Evans, J. F., and Habenicht, A. J. R. (2003) Differential leukotriene receptor expression and calcium responses in endothelial cells and macrophages indicate 5-lipoxygenase-dependent circuits of inflammation and atherogenesis. *Arteriosclerosis Thrombosis and Vascular Biology* **23**, E32-E36
154. Khor, Y. H., Teoh, A. K. Y., Lam, S. M., Mo, D. C. Q., Weston, S., Reid, D. W., and Walters, E. H. (2009) Increased vascular permeability precedes cellular inflammation as asthma control deteriorates. *Clinical and Experimental Allergy* **39**, 1659-1667
155. Orange, R. P., and Austen, K. F. (1969) Slow reacting substance of anaphylaxis in the rat. *Movat, Henry Z. (Edited by). Cellular and Humoral Mechanisms in Anaphylaxis and Allergy. Ix + 288p. Illus. S. Karger: Basel, Switzerland and New York, N.Y., U.S.A*, 196-206
156. Woodward, D. F., Weichman, B. M., Gill, C. A., and Wasserman, M. A. (1983) The effect of synthetic leukotrienes on tracheal microvascular permeability. *Prostaglandins* **25**, 131-142
157. Joris, I., Majno, G., Corey, E. J., and Lewis, R. A. (1987) The mechanism of vascular leakage induced by leukotriene-E4- endothelial contraction. *American Journal of Pathology* **126**, 19-24
158. Kanaoka, Y., Maekawa, A., Penrose, J. F., Austen, K. F., and Lam, B. K. (2001) Attenuated zymosan-induced peritoneal vascular permeability and IgE dependent passive cutaneous anaphylaxis in mice lacking leukotriene C-4 synthase. *Journal of Biological Chemistry* **276**, 22608-22613
159. Maekawa, A., Austen, K. F., and Kanaoka, Y. (2002) Targeted gene disruption reveals the role of cysteinyl leukotriene 1 receptor in the enhanced vascular permeability of mice undergoing acute inflammatory responses. *Journal of Biological Chemistry* **277**, 20820-20824
160. Barnes, P. J. (2007) New molecular targets for the treatment of neutrophilic diseases. *Journal of Allergy and Clinical Immunology* **119**, 1055-1062
161. Noveral, J. P., and Grunstein, M. M. (1992) Role and mechanism of thromboxane-induced proliferation of cultured airways smooth-muscle cells. *American Journal of Physiology* **263**, L555-L561
162. Wang, C. G., Du, T., Xu, L. J., and Martin, J. G. (1993) Role of leukotriene-D(4) in allergen-induced increases in airway smooth-muscle in the rat. *American Review of Respiratory Disease* **148**, 413-417
163. Accomazzo, M. R., Rovati, G. E., Vigano, T., Hernandez, A., Bonazzi, A., Bolla, M., Fumagalli, F., Viappiani, S., Galbiati, E., Ravasi, S., Albertoni, C., Di Luca, M., Caputi, A., Zannini, P., Chiesa, G., Villa, A. M., Doglia, S. M., Folco, G., and Nicosia, S. (2001) Leukotriene D(4)-induced activation of smooth-muscle cells from human bronchi is partly Ca(2+)-independent. *American Journal of Respiratory and Critical Care Medicine* **163**, 266-272
164. Panettieri, R. A., Tan, E. M. L., Ciocca, V., Luttmann, M. A., Leonard, T. B., and Hay, D. W. P. (1998) Effects of LTD4 on human airway smooth muscle cell proliferation, matrix expression, and contraction in vitro: Differential sensitivity to cysteinyl leukotriene receptor antagonists. *American Journal of Respiratory Cell and Molecular Biology* **19**, 453-461
165. Cohen, P., Noveral, J. P., Bhala, A., Nunn, S. E., Herrick, D. J., and Grunstein, M. M. (1995) Leukotriene D-4 facilitates airway smooth-muscle cell-proliferation via modulation og the IGF axis. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **269**, L151-L157
166. Foster, A., and Chan, C. C. (1991) Peptide leukotriene involvment in pulmonary eosinophil migration upon antigen challenge in the actively sensitized guinea-pig. *International Archives of Allergy and Applied Immunology* **96**, 279-284

167. Turner, C. R., Smith, W. B., Andresen, C. J., Swindell, A. C., and Watson, J. W. (1994) Leukotriene-D4 receptor antagonism reduced airway hyperresponsiveness in monkeys. *Pulmonary Pharmacology* **7**, 49-58
168. Landgraf, R. G., Nossi, D. F., Sirois, P., and Jancar, S. (2007) Prostaglandins, leukotrienes and PAF selectively modulate lymphocyte subset and eosinophil infiltration into the airways in a murine model of asthma. *Prostaglandins Leukotrienes and Essential Fatty Acids* **77**, 163-172
169. Underwood, S., Foster, M., Raeburn, D., Bottoms, S., and Karlsson, J. A. (1995) Time-course of antigen-induced airway inflammation in the guinea-pig and its relationship to airway hyperresponsiveness. *European Respiratory Journal* **8**, 2104-2113
170. Paruchuri, S., Tashimo, H., Feng, C. L., Maekawa, A., Xing, W., Jiang, Y. F., Kanaoka, Y., Conley, P., and Boyce, J. A. (2009) Leukotriene E(4)-induced pulmonary inflammation is mediated by the P2Y(12) receptor. *Journal of Experimental Medicine* **206**, 2543-2555
171. Laitinen, A., Lindqvist, A., Halme, M., Altraja, A., and Laitinen, L. A. (2005) Leukotriene E-4-induced persistent eosinophilia and airway obstruction are reversed by zafirlukast in patients with asthma. *Journal of Allergy and Clinical Immunology* **115**, 259-265
172. Gauvreau, G. M., Parameswaran, K. N., Watson, R. M., and O'Byrne, P. M. (2001) Inhaled leukotriene E-4, but not leukotriene D-4, increased airway inflammatory cells in subjects with atopic asthma. *American Journal of Respiratory and Critical Care Medicine* **164**, 1495-1500
173. Hemelaers, L., Henket, M., Sele, J., Bureau, F., and Louis, R. (2006) Cysteinyl-leukotrienes contribute to sputum eosinophil chemotactic activity in asthmatics. *Allergy* **61**, 136-139
174. Fregonese, L., Silvestri, M., Sabatini, F., and Rossi, G. A. (2002) Cysteinyl leukotrienes induce human eosinophil locomotion and adhesion molecule expression via a CysLT(1) receptor-mediated mechanism. *Clinical and Experimental Allergy* **32**, 745-750
175. Nagata, M., Saito, K., Tsuchiya, K., and Sakamoto, Y. (2002) Leukotriene D-4 upregulates eosinophil adhesion via the cysteinyl leukotriene 1 receptor. *Journal of Allergy and Clinical Immunology* **109**, 676-680
176. Carroll, N. G., Mutavdzic, S., and James, A. L. (2002) Increased mast cells and neutrophils in submucosal mucous glands and mucus plugging in patients with asthma. *Thorax* **57**, 677-682
177. Liu, Y. C., Khawaja, A. M., and Rogers, D. F. (1998) Effects of the cysteinyl leukotriene receptor antagonists pranlukast and zafirlukast on tracheal mucus secretion in ovalbumin-sensitized guinea-pigs in vitro. *British Journal of Pharmacology* **124**, 563-571
178. Hoffstein, S. T., Malo, P. E., Bugelski, P., and Wheeldon, E. B. (1990)Leukotriene-D4 (LTD4) induces mucus secretion from goblet cells in the guinea-pig respiratory epithelium. *Experimental Lung Research* **16**, 711-725
179. Shelhamer, J. H., Marom, Z., Sun, F., Bach, M. K., and Kaliner, M. (1982) The effects of arachoids and leukotrienes on the release of mucus from human airways. *Chest* **81**, S36-S37
180. Marom, Z. V. I., Shelhamer, J. H., Bach, M. K., Morton, D. R., and Kaliner, M. (1982) Slow-reacting substances, leukotriene-C4 and leukotriene-D4, increase the release of mucus from human airways *in vitro*. *American Review of Respiratory Disease* **126**, 449-451
181. Coles, S. J., Neill, K. H., Reid, L. M., Austen, K. F., Nii, Y., Corey, E. J., and Lewis, R. A. (1983) Effects of leukotriene-C4 and leukotriene-D4 on glycoprotein and lysozyme secretion by human bronchial-mucosa. *Prostaglandins* **25**, 155-170

182. Ahmed, T., Garrigo, J., and Danta, I. (1993) Preventing bronchoconstriction in exercise-induced asthma with inhaled heparin. *New England Journal of Medicine* **329**, 90-95
183. Bisgaard, H., and Pedersen, M. (1987) SRS-A leukotrienes decrease the activity of human respiratory cilia. *Clinical Allergy* **17**, 95-103
184. Joki, S., Saano, V., Koskela, T., Toskala, E., Bray, M. A., and Nuutinen, J. (1996) Effect of leukotriene D4 on ciliary activity in human, guinea-pig and rat respiratory mucosa. *Pulmonary Pharmacology & Therapeutics* **9**, 231-238
185. Dartt, D. A., Hodges, R. R., Li, D., Shatos, M. A., Lashkari, K., and Serhan, C. N. (2011) Conjunctival Goblet Cell Secretion Stimulated by Leukotrienes Is Reduced by Resolvins D1 and E1 To Promote Resolution of Inflammation. *Journal of Immunology* **186**, 4455-4466
186. Berger, W., De Chandt, M. T. M., and Cairns, C. B. (2007) Zileuton: clinical implications of 5-Lipoxygenase inhibition in severe airway disease. *International Journal of Clinical Practice* **61**, 663-676
187. Wasfi, Y. S., Villaran, C., de Tilleghem, C. L. B., Smugar, S. S., Hanley, W. D., Reiss, T. F., and Knorr, B. A. (2012) The efficacy and tolerability of MK-0633, a 5-lipoxygenase inhibitor, in chronic asthma. *Respiratory Medicine* **106**, 34-46
188. Follows, R. M. A., Snowise, N. G., Ho, S.-Y., Ambery, C. L., Smart, K., and McQuade, B. A. (2013) Efficacy, safety and tolerability of GSK2190915, a 5-lipoxygenase activating protein inhibitor, in adults and adolescents with persistent asthma: a randomised dose-ranging study. *Respiratory Research* **14**:54
189. (2013) British guideline on the management of asthma. www.brit-thoracic.org.uk
190. Kraft, M., Cairns, C. B., Ellison, M. C., Pak, J., Irvin, C., and Wenzel, S. (2006) Improvements in distal lung function correlate with asthma symptoms after treatment with oral montelukast. *Chest* **130**, 1726-1732
191. Reiss, T. F., Chervinsky, P., Dockhorn, R. J., Shingo, S., Seidenberg, B., Edwards, T. B., and Montelukast Clinical Res Study, G. (1998) Montelukast, a once-daily leukotriene receptor antagonist, in the treatment of chronic asthma - A multicenter, randomized, double-blind trial. *Archives of Internal Medicine* **158**, 1213-1220
192. Zhang, H. P., Jia, C. E., Lv, Y., Gibson, P. G., and Wang, G. (2014) Montelukast for prevention and treatment of asthma exacerbations in adults: Systematic review and meta-analysis. *Allergy and asthma proceedings : the official journal of regional and state allergy societies* **35**, 278-287
193. Garcia, M. L. G., Wahn, U., Gilles, L., Swern, A., Tozzi, C. A., and Polos, P. (2005) Montelukast, compared with fluticasone, for control of asthma among 6- to 14-year-old patients with mild asthma: The MOSAIC Study. *Pediatrics* **116**, 360-369
194. Szefler, S. J., Carlsson, L.-G., Uryniak, T., and Baker, J. W. (2013) Budesonide inhalation suspension versus montelukast in children aged 2 to 4 years with mild persistent asthma. *The journal of allergy and clinical immunology. In practice* **1**, 58-64
195. Shah, M. B., Gohil, J., Khapekar, S., and Dave, J. (2014) Montelukast versus Budesonide as a First Line Preventive Therapy in Mild Persistent Asthma in 2 to 18 y. *Indian journal of pediatrics* **81**, 655-659
196. Tomari, S., Shimoda, T., Kawano, T., Mitsuta, K., Obase, Y., Fukushima, C., Matsuse, H., and Kohno, S. (2001) Effects of pranlukast, a cysteinyl leukotriene receptor 1 antagonist, combined with inhaled beclomethasone in patients with moderate or severe asthma. *Annals of Allergy Asthma & Immunology* **87**, 156-161
197. Price, D. B., Hernandez, D., Magyar, P., Fiterman, J., Beeh, K. M., James, I. G., Konstantopoulos, S., Rojas, R., van Noord, J. A., Pons, M., Gilles, L., Leff, J. A., and Grp, C. S. (2003) Randomised controlled trial of montelukast plus inhaled budesonide versus double dose inhaled budesonide in adult patients with asthma. *Thorax* **58**, 211-216

198. Barnes, N., Laviolette, M., Allen, D., Flood-Page, P., Hargreave, F., Corris, P., O'Connor, B. J., Tate, H., Parker, D., and Pavord, I. (2007) Effects of montelukast compared to double dose budesonide on airway inflammation and asthma control. *Respiratory Medicine* **101**, 1652-1658
199. Kelly, H. W. (2003) Potential adverse effects of the inhaled corticosteroids. *Journal of Allergy and Clinical Immunology* **112**, 469-478
200. Carraro, S., Corradi, M., Zanconato, S., Alinovi, R., Pasquale, M. F., Zacchello, F., and Baraldi, E. (2005) Exhaled breath condensate cysteinyl leukotrienes are increased in children with exercise-induced bronchoconstriction. *Journal of Allergy and Clinical Immunology* **115**, 764-770
201. Reiss, T. F., Hill, J. B., Harman, E., Zhang, J., Tanaka, W. K., Bronsky, E., Guerreiro, D., and Hendeles, L. (1997) Increased urinary excretion of LTE4 after exercise and attenuation of exercise-induced bronchospasm by montelukast, a cysteinyl leukotriene receptor antagonist. *Thorax* **52**, 1030-1035
202. Bronsky, E. A., Kemp, J. P., Zhang, J., Guerreiro, D., and Reiss, T. F. (1997) Dose-related protection of exercise bronchoconstriction by montelukast, a cysteinyl leukotriene-receptor antagonist, at the end of a once-daily dosing interval. *Clinical Pharmacology & Therapeutics* **62**, 556-561
203. Christie, P. E., Tagari, P., Fordhutchinson, A. W., Charlesson, S., Chee, P., Arm, J. P., and Lee, T. H. (1991) Urinary leukotriene-E4 concentrations increase after aspirin challenge in aspirin-sensitive asthmatic subjects. *American Review of Respiratory Disease* **143**, 1025-1029
204. Dahlen, S. E., Malmstrom, K., Nizankowska, E., Dahlen, B., Kuna, P., Kowalski, M., Lumry, W. R., Picado, C., Stevenson, D. D., Bousquet, J., Pauwels, R., Holgate, S. T., Shahane, A., Zhang, J., Reiss, T. F., and Szczeklik, A. (2002) Improvement of asprinin-intolerant asthma by montelukast, a leukotriene antagonist - A randomized, double-blink, placebo-controlled trial. *American Journal of Respiratory and Critical Care Medicine* **165**, 9-14
205. Drazen, J., Lewis, R., Austen, K., and Corey, E. (1983) *Pulmonary pharmacology of the SRS-A leukotrienes*, Plenum, New York, **54**, 125-134
206. Lee, T. H., Austen, K. F., Corey, E. J., and Drazen, J. M. (1984) Leukotriene E-4-induced airway hyperresponsiveness of guinea-pig tracheal smooth-muscle to histamine and evidence for 3 separate sulfidopeptide leukotriene receptors. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences* **81**, 4922-4925
207. Jacques, C. A. J., Spur, B. W., Johnson, M., and Lee, T. H. (1991) The mechanism of LTE4-induced histamine hyperresponsiveness in guinea-pig tracheal and human bronchial smooth-muscle, *in vitro*. *British Journal of Pharmacology* **104**, 859-866
208. Jacques, C. A. J., Spur, B. W., Johnson, M., and Lee, T. H. (1992) The effect of epithelium removal on leukotriene E4-induced histamine hyperresponsiveness in guinea-pig tracheal smooth-muscle. *British Journal of Pharmacology* **106**, 556-562
209. Snetkov, V. A., Hapgood, K. J., McVicker, C. G., Lee, T. H., and Ward, J. P. T. (2001) Mechanisms of leukotriene D-4-induced constriction in human small bronchioles. *British Journal of Pharmacology* **133**, 243-252
210. Davidson, A. B., Lee, T. H., Scanlon, P. D., Solway, J., McFadden, E. R., Ingram, R. H., Corey, E. J., Austen, K. F., and Drazen, J. M. (1987) Bronchoconstrictor effects of leukotriene-E4 in normal and asthmatic subjects. *American Review of Respiratory Disease* **135**, 333-337
211. Arm, J. P., Ohickey, S. P., Hawksworth, R. J., Fong, C. Y., Crea, A. E. G., Spur, B. W., and Lee, T. H. (1990) Asthmatic airways have a disproportionate hyperresponsiveness to LTE4, as compared with normal airways, but not to LTC4, LTD4, metacholine and histamine. *American Review of Respiratory Disease* **142**, 1112-1118
212. Arm, J. P., Ohickey, S. P., Spur, B. W., and Lee, T. H. (1989) Airway responsiveness to histamine and leukotriene-E4 in subjects with aspirin-induced asthma. *American Review of Respiratory Disease* **140**, 148-153

213. Maltby, N. H., Taylor, G. W., Ritter, J. M., Moore, K., Fuller, R. W., and Dollery, C. T. (1990) Leukotriene-C4 elimination and metabolism in man. *Journal of Allergy and Clinical Immunology* **85**, 3-9
214. Kumlin, M., Dahlen, B., Bjorck, T., Zetterstrom, O., Granstrom, E., and Dahlen, S. E. (1992) Urinary-excretion of leukotriene-E4 and 11-dehydro-thromboxane-B2 in response to bronchial provocations with allergens, aspirin, leukotriene-D4, and histamine in asthmatics. *American Review of Respiratory Disease* **146**, 96-103
215. Knapp, H. R., Sladek, K., and Fitzgerald, G. A. (1992) Increased excretion of leukotriene-E4 during aspirin-induced asthma. *Journal of Laboratory and Clinical Medicine* **119**, 48-51
216. Yoshikawa, K., Matsui, E., Inoue, R., Kaneko, H., Teramoto, T., Aoki, M., Kasahara, K., Shinoda, S., Fukutomi, O., and Kondo, N. (2004) Urinary leukotriene E4 and 11-dehydro-thromboxane B2 excretion in children with bronchial asthma. *Allergology International* **53**, 127-134
217. Laitinen, L. A., Laitinen, A., Haahtela, T., Vilkka, V., Spur, B. W., and Lee, T. H. (1993) Leukotriene-E(4) and granulocytic infiltration into asthmatic airways. *Lancet* **341**, 989-990
218. Maekawa, A., Kanaoka, Y., Xing, W., and Austen, K. F. (2008) Functional recognition of a distinct receptor preferential for leukotriene E-4 in mice lacking the cysteinyl leukotriene 1 and 2 receptors. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 16695-16700
219. Laidlaw, T. M., Steinke, J. W., Tinana, A. M., Feng, C., Xing, W., Lam, B. K., Paruchuri, S., Boyce, J. A., and Borish, L. (2011) Characterization of a novel human mast cell line that responds to stem cell factor and expresses functional Fc is an element of RI. *Journal of Allergy and Clinical Immunology* **127**, 815-822
220. Hollopeter, G., Jantzen, H. M., Vincent, D., Li, G., England, L., Ramakrishnan, V., Yang, R. B., Nurden, P., Nurden, A., Julius, D., and Conley, P. B. (2001) Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature* **409**, 202-207
221. Foster, C. J., Prosser, D. M., Agans, J. M., Zhai, Y., Smith, M. D., Lachowicz, J. E., Zhang, F. L., Gustafson, E., Monsma, F. J., Wiekowski, M. T., Abbondanzo, S. J., Cook, D. N., Bayne, M. L., Lira, S. A., and Chintala, M. S. (2001) Molecular identification and characterization of the platelet ADP receptor targeted by thienopyridine antithrombotic drugs. *Journal of Clinical Investigation* **107**, 1591-1598
222. Zhang, F. L., Luo, L., Gustafson, E., Lachowicz, J., Smith, M., Qiao, X. D., Liu, Y. H., Chen, G. D., Pramanik, B., Laz, T. M., Palmer, K., Bayne, M., and Monsma, F. J. (2001) ADP is the cognate ligand for the orphan G protein-coupled receptor SP1999. *Journal of Biological Chemistry* **276**, 8608-8615
223. Fabre, J. E., Nguyen, M. T., Latour, A., Keifer, J. A., Audoly, L. P., Coffman, T. M., and Koller, B. H. (1999) Decreased platelet aggregation, increased bleeding time and resistance to thromboembolism in P2Y(1)-deficient mice. *Nature Medicine* **5**, 1199-1202
224. Hardy, A. R., Jones, M. L., Mundell, S. J., and Poole, A. W. (2004) Reciprocal cross-talk between P2Y(1) and P2Y(12) receptors at the level of calcium signaling in human platelets. *Blood* **104**, 1745-1752
225. Burnstock, G., and Knight, G. E. (2004) Cellular distribution and functions of P2 receptor subtypes in different systems. *International Review of Cytology - a Survey of Cell Biology, Vol. 240* **240**, 31-304
226. Burnstock, G. (1976) Purinergic receptors. *Journal of Theoretical Biology* **62**, 491-503
227. Burnstock, G. (1978) A basis for distinguishing 2 types of purinergic receptor. *Straub, Ralph W. and Liana Bolis (Ed.). Cell Membrane Receptors for Drugs and Hormones: a Multidisciplinary Approach. Xv+356p. Illus. Raven Press: New York, N.Y., USA. Isbn 0-89004-227-6*, 107-118
228. Burnstock, G., and Kennedy, C. (1985) Is there a basis for distinguishing 2 types of P2-purinoceptor. *General Pharmacology* **16**, 433-440

229. Abbracchio, M. P., Burnstock, G., Boeynaems, J.-M., Barnard, E. A., Boyer, J. L., Kennedy, C., Knight, G. E., Fumagalli, M., Gachet, C., Jacobson, K. A., and Weisman, G. A. (2006) International union of pharmacology LVIII: Update on the P2Y G protein-coupled nucleotide receptors: From molecular mechanisms and pathophysiology to therapy. *Pharmacological Reviews* **58**, 281-341
230. Yokomizo, T., Izumi, T., Chang, K., Takuwa, Y., and Shimizu, T. (1997) A G-protein-coupled receptor for leukotriene B-4 that mediates chemotaxis. *Nature* **387**, 620-624
231. Herold, C. L., Li, Q., Schachter, J. B., Harden, T. K., and Nicholas, R. A. (1997) Lack of nucleotide-promoted second messenger signaling responses in 1321N1 cells expressing the proposed P2Y receptor, p2y7. *Biochemical and Biophysical Research Communications* **235**, 717-721
232. He, W. H., Miao, F. J. P., Lin, D. C. H., Schwandner, R. T., Wang, Z. L., Gao, J. H., Chen, J. L., Tian, H., and Ling, L. (2004) Citric acid cycle intermediates as ligands for orphan G-protein-coupled receptors. *Nature* **429**, 188-193
233. Nonaka, Y., Hiramoto, T., and Fujita, N. (2005) Identification of endogenous surrogate ligands for human P2Y12 receptors by in silico and in vitro methods. *Biochem Biophys Res Commun* **337**, 281-288
234. Southern, C., Cook, J. M., Neetoo-Isseljee, Z., Taylor, D. L., Kettleborough, C. A., Merritt, A., Bassoni, D. L., Raab, W. J., Quinn, E., Wehrman, T. S., Davenport, A. P., Brown, A. J., Green, A., Wigglesworth, M. J., and Rees, S. (2013) Screening beta-Arrestin Recruitment for the Identification of Natural Ligands for Orphan G-Protein-Coupled Receptors. *Journal of Biomolecular Screening* **18**, 599-609
235. Tokonami, N., Morla, L., Centeno, G., Mordasini, D., Ramakrishnan, S. K., Nikolaeva, S., Wagner, C. A., Bonny, O., Houillier, P., Doucet, A., and Firsov, D. (2013) alpha-Ketoglutarate regulates acid-base balance through an intrarenal paracrine mechanism. *Journal of Clinical Investigation* **123**, 3166-3171
236. Kanaoka, Y., Maekawa, A., and Austen, K. F. (2013) Identification of GPR99 Protein as a Potential Third Cysteinyl Leukotriene Receptor with a Preference for Leukotriene E-4 Ligand. *Journal of Biological Chemistry* **288**, 10967-10972
237. Maekawa, A., Kanaoka, Y., Xing, W., and Austen, K. (2008) Functional recognition of a distinct receptor preferential for leukotriene E_4 in mice lacking the cysteinyl leukotriene 1 and 2 receptors. *Proceedings of the National Academy of Sciences of the USA* **105**, 16695-16700
238. Heise, C. E., O'Dowd, B. F., Figueroa, D. J., Sawyer, N., Nguyen, T., Im, D.-S., Stocco, R., Bellefeuille, J. N., Abramovitz, M., Cheng, R., Williams, D. L., Jr., Zeng, Z., Liu, Q., Ma, L., Clements, M. K., Coulombe, N., Liu, Y., Austin, C. P., George, S. R., O'Neill, G. P., Metters, K. M., Lynch, K. R., and Evans, J. F. (2000) Characterization of the human cysteinyl leukotriene 2 receptor. *Journal of Biological Chemistry* **275**, 30531-30536
239. Lynch, K., O'Neill, G., Liu, G., Im, D., Sawyer, N., Metters, K., Coulombe, N., Abramovitz, M., Figueroa, D., Zeng, Z., Connolly, B., Bai, C., Austin, C., Chateauneuf, A., Stocco, R., Greig, G., Kargman, S., Hooks, S., Hosfield, H., Williams, D., Ford-Hutchinson, A., Caskey, C., and Evans, J. (1999) Characterization of the human cysteinyl leukotriene $CysLT_1$ receptor. *Nature* **399**, 789-793
240. Offermanns, S., and Simon, M. I. (1995) G-alpha(15) and G-alpha(16) couple a wide variety of receptors to phospholipase-C. *Journal of Biological Chemistry* **270**, 15175-15180
241. Luttrell, L. M., and Lefkowitz, R. J. (2002) The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. *Journal of Cell Science* **115**, 455-465
242. Gresele, P., Dottorini, M., Selli, M. L., Iannacci, L., Canino, S., Todisco, T., Romano, S., Crook, P., Page, C. P., and Nenci, G. G. (1993) Altered platelet-function associated with the bronchial hyperresponsiveness accompanying nocturnal asthma. *Journal of Allergy and Clinical Immunology* **91**, 894-902

243. Kowal, K., Pampuch, A., Kowal-Bielecka, O., DuBuske, L. M., and Bodzenta-Lukaszyk, A. (2006) Platelet activation in allergic asthma patients during allergen challenge with Dermatophagoides pteronyssinus. *Clinical and Experimental Allergy* **36**, 426-432
244. Yamamoto, H., Nagata, M., Tabe, K., Kimura, I., Kiuchi, H., Sakamoto, Y., Yamamoto, K., and Dohi, Y. (1993) The evidence of platelet activation in bronchial-asthma. *Journal of Allergy and Clinical Immunology* **91**, 79-87
245. Pitchford, S. C., Yano, H., Lever, R., Riffo-Vasquez, Y., Ciferri, S., Rose, M. J., Giannini, S., Momi, S., Spina, D., O'Connor, B., Gresele, P., and Page, C. P. (2003) Platelets are essential for leukocyte recruitment in allergic inflammation. *Journal of Allergy and Clinical Immunology* **112**, 109-118
246. Pitchford, S. C., Riffo-Vasquez, Y., Sousa, A., Momi, S., Gresele, P., Spina, D., and Page, C. P. (2004) Platelets are necessary for airway wall remodeling in a murine model of chronic allergic inflammation. *Blood* **103**, 639-647
247. Pitchford, S. C., and Page, C. P. (2006) Platelet activation in asthma: integral to the inflammatory response. *Clinical and Experimental Allergy* **36**, 399-401
248. Pitchford, S. C., Momi, S., Baglioni, S., Casali, L., Giannini, S., Rossi, R., Page, C. P., and Gresele, P. (2008) Allergen induces the migration of platelets to lung tissue in allergic asthma. *American Journal of Respiratory and Critical Care Medicine* **177**, 604-612
249. Matsumoto, K., Imagawa, S., Obara, N., Suzuki, N., Takahashi, S., Nagasawa, T., and Yamamoto, M. (2006) 2-Oxoglutarate downregulates expression of vascular endothelial growth factor and erythropoietin through decreasing hypoxia-inducible factor-1 alpha and inhibits angiogenesis. *Journal of Cellular Physiology* **209**, 333-340
250. Rzeski, W., Walczak, K., Juszczak, M., Langner, E., Pozarowski, P., Kandefer-Szerszen, M., and Pierzynowski, S. G. (2012) Alpha-ketoglutarate (AKG) inhibits proliferation of colon adenocarcinoma cells in normoxic conditions. *Scandinavian Journal of Gastroenterology* **47**, 565-571
251. Pugh, C. W., Orourke, J. F., Nagao, M., Gleadle, J. M., and Ratcliffe, P. J. (1997) Activation of hypoxia-inducible factor-1; Definition of regulatory domains within the alpha subunit. *Journal of Biological Chemistry* **272**, 11205-11214
252. Lev, S., Yarden, Y., and Givol, D. (1992) Dimerization and activation of the Kit receptor by monovalent and bivalent binding of the stem-cell factor. *Journal of Biological Chemistry* **267**, 15970-15977
253. Blumejensen, P., Claessonwelsh, L., Siegbahn, A., Zsebo, K. M., Westermark, B., and Heldin, C. H. (1991) Activation of the human c-Kit product by ligand-induced dimerization mediates circular actin reorganization and chemotaxis. *Embo Journal* **10**, 4121-4128
254. Jiang, Y., Kanaoka, Y., Feng, C., Nocka, K., Rao, S., and Boyce, J. A. (2006) Cutting edge: Interleukin 4-dependent mast cell proliferation requires Autocrine/Intracrine cysteinyl leukotriene-induced signaling. *Journal of Immunology* **177**, 2755-2759
255. Butterfield, J. H., Weiler, D., Dewald, G., and Gleich, G. J. (1988) Establishment of an immature mast-cell line from a patient with mast-cell leukemia. *Leukemia Research* **12**, 345-355
256. Guhl, S., Babina, M., Neou, A., Zuberbier, T., and Artuc, M. (2010) Mast cell lines HMC-1 and LAD2 in comparison with mature human skin mast cells - drastically reduced levels of tryptase and chymase in mast cell lines. *Experimental Dermatology* **19**, 845-847
257. Paruchuri, S., Jiang, Y., Feng, C., Francis, S. A., Plutzky, J., and Boyce, J. A. (2008) Leukotriene E(4) activates peroxisome proliferator-activated receptor gamma and induces prostaglandin D(2) generation by human mast cells. *Journal of Biological Chemistry* **283**, 16477-16487

258. Ryder, C., McColl, K., Zhong, F., and Distelhorst, C. W. (2012) Acidosis Promotes Bcl-2 Family-mediated Evasion of Apoptosis Involvment of acid-sensing G protein-coupled receptor GPR65 signaling to MEK/ERK. *Journal of Biological Chemistry* **287**, 27863-27875
259. Xu, H., Chen, X., Huang, J., Deng, W., Zhong, Q., Yue, C., Wang, P., and Huang, Z. (2013) Identification of GPR65, a novel regulator of matrix metalloproteinases using high through-put screening. *Biochemical and Biophysical Research Communications* **436**, 96-103
260. Subramanian, H., Gupta, K., Guo, Q., Price, R., and Ali, H. (2011) Mas-related Gene X2 (MrgX2) Is a Novel G Protein-coupled Receptor for the Antimicrobial Peptide LL-37 in Human Mast Cells resistance to receptor phosphorylation, desensitization, and internalization. *Journal of Biological Chemistry* **286**, 44739-44749
261. Subramanian, H., Gupta, K., Lee, D., Bayir, A. K., Ahn, H., and Ali, H. (2013) beta-Defensins Activate Human Mast Cells via Mas-Related Gene X2. *Journal of Immunology* **191**, 345-352
262. Reiss, T. F., Altman, L. C., Chervinsky, P., Bewtra, A., Stricker, W. E., Noonan, G. P., Kundu, S., and Zhang, J. (1996) Effects of montelukast (MK-0476), a new potent cysteinyl leukotriene (LDT(4)) receptor antagonist, in patients with chronic asthma. *Journal of Allergy and Clinical Immunology* **98**, 528-534
263. Mamedova, L., Capra, V., Accomazzo, M. R., Gao, Z. G., Ferrario, S., Fumagalli, M., Abbracchio, M. P., Rovati, G. E., and Jacobson, K. A. (2005) CysLT(1) leukotriene receptor antagonists inhibit the effects of nucleotides acting at P2Y receptors. *Biochemical Pharmacology* **71**, 115-125
264. Anderson, R., Theron, A. J., Gravett, C. M., Steel, H. C., Tintinger, G. R., and Feldman, C. (2009) Montelukast inhibits neutrophil pro-inflammatory activity by a cyclic AMP-dependent mechanism. *British Journal of Pharmacology* **156**, 105-115
265. Robinson, A. J., Kashanin, D., O'Dowd, F., Williams, V., and Walsh, G. M. (2008) Montelukast inhibition of resting and GM-CSF-stimulated eosinophil adhesion to VCAM-1 under flow conditions appears independent of cysLT(1)R antagonism. *Journal of Leukocyte Biology* **83**, 1522-1529
266. Tahan, F., Jazrawi, E., Moodley, T., Rovati, G. E., and Adcock, I. M. (2008) Montelukast inhibits tumour necrosis factor-alpha-mediated interleukin-8 expression through inhibition of nuclear factor-kappa B p65-associated histone acetyltransferase activity. *Clinical and Experimental Allergy* **38**, 805-811
267. Woszczek, G., Chen, L. Y., Alsaaty, S., Nagineni, S., and Shelhamer, J. H. (2010) Concentration-Dependent Noncysteinyl Leukotriene Type 1 Receptor-Mediated Inhibitory Activity of Leukotriene Receptor Antagonists. *Journal of Immunology* **184**, 2219-2225
268. Zhang, J., Migita, O., Koga, M., Shibasaki, M., Arinami, T., and Noguchi, E. (2006) Determination of structure and transcriptional regulation of CYSLTR1 and an association study with asthma and rhinitis. *Pediatric Allergy and Immunology* **17**, 242-249
269. Duroudier, N. P., Strachan, D. P., Blakey, J. D., and Hall, I. P. (2009) Association of the cysteinyl leukotriene receptor 1 gene with atopy in the British 1958 birth cohort. *Journal of Allergy and Clinical Immunology* **124**, 566-572
270. Kim, S. H., Oh, J. M., Kim, Y. S., Palmer, L. J., Suh, C. H., Nahm, D. H., and Park, H. S. (2006) Cysteinyl leukotriene receptor 1 promoter polymorphism is associated with aspirin-intolerant asthma in males. *Clinical and Experimental Allergy* **36**, 433-439
271. Arriba-Mendez, S., Sanz, C., Isidoro-Garcia, M., Davild, I., Laffond, E., Horeno, E., Avila, C., and Lorente, F. (2006) 927T > C polymorphism of the cysteinyl-leukotriene type-1 receptor (CYSLTR1) gene in children with asthma and atopic dermatitis. *Pediatric Allergy and Immunology* **17**, 323-328

272. Hao, L., Sayers, I., Cakebread, J. A., Barton, S. J., Beghe, B., Holgate, S. T., Sampson, A. P., and Holloway, J. W. (2006) The cysteinyl-leukotriene type 1 receptor polymorphism 927T/C is associated with atopy severity but not with asthma. *Clinical and Experimental Allergy* **36**, 735-741
273. Thompson, M. D., Capra, V., Takasaki, J., Maresca, G., Rovati, G. E., Slutsky, A. S., Lilly, C., Zamel, N., Burnham, W. M., Cole, D. E., and Siminovitch, K. A. (2007) A functional G300S variant of the cysteinyl leukotriene 1 receptor is associated with atopy in a Tristan da Cunha isollate. *Pharmacogenetics and Genomics* **17**, 539-549
274. Sokolowska, M., Wodz-Naskiewicz, K., Cieslak, M., Seta, K., Bednarek, A. K., and Pawliczak, R. (2009) Variable expression of cysteinyl leukotriene type I receptor splice variants in asthmatic females with different promoter haplotypes. *Bmc Immunology* **10**:63
275. Duan, J. B., Wainwright, M. S., Comeron, J. M., Saitou, N., Sanders, A. R., Gelernter, J., and Gejman, P. V. (2003) Synonymous mutations in the human dopamine receptor D2 (DRD2) affect mRNA stability and synthesis of the receptor. *Human Molecular Genetics* **12**, 205-216
276. Mong, S., Wu, H. L., Clark, M. A., Gleason, J. G., and Crooke, S. T. (1986) Leukotriene-D4 receptor-mediated synthesis and release of arachidonic-acid metabolites in guinea-pig lung-induction of thromboxane and prostacyclin biosynthesis by leukotriene-D4. *Journal of Pharmacology and Experimental Therapeutics* **239**, 63-70
277. Capra, V., Ravasi, S., Accomazzo, M. R., Parenti, M., and Rovati, G. E. (2004) CysLT(1) signal transduction in differentiated U937 cells involves the activation of the small GTP-binding protein Ras. *Biochemical Pharmacology* **67**, 1569-1577
278. Funk, C. D. (2001) Prostaglandins and leukotrienes: Advances in eicosanoid biology. *Science* **294**, 1871-1875
279. Boehme, I., and Beck-Sickinger, A. G. (2009) Illuminating the life of GPCRs. *Cell Communication and Signaling* **7**:16
280. Bandeira-Melo, C., Hall, J. C., Penrose, J. F., and Weller, P. F. (2002) Cysteinyl leukotrienes induce IL-4 release from cord blood-derived human eosinophils. *Journal of Allergy and Clinical Immunology* **109**, 975-979
281. Peres, C. M., Aronoff, D. M., Serezani, C. H., Flamand, N., Faccioli, L. H., and Peters-Golden, M. (2007) Specific leukotriene receptors couple to distinct G proteins to effect stimulation of alveolar macrophage host defense functions. *Journal of Immunology* **179**, 5454-5461
282. Saussy, D. L., Sarau, H. M., Foley, J. J., Mong, S., and Crooke, S. T. (1989) Mechanisms of leukotriene-E4 partial agonist activity at leukotriene D4 receptors in differentiated U-937-cells. *Journal of Biological Chemistry* **264**, 19845-19855
283. Thodeti, C. K., Nielsen, C. K., Paruchuri, S., Larsson, C., and Sjolander, A. (2001) The epsilon isoform of protein kinase C is involved in regulation of the LTD4-induced calcium signal in human intestinal epithelial cells. *Experimental Cell Research* **262**, 95-103
284. Kondeti, V., Duah, E., Al-Azzam, N., Thodeti, C. K., Boyce, J. A., and Paruchuri, S. (2013) Differential Regulation of Cysteinyl Leukotriene Receptor Signaling by Protein Kinase C in Human Mast Cells. *Plos One* **8(8)**: e71536
285. Alblas, J., Vanetten, I., Khanum, A., and Moolenaar, W. H. (1995) C-terminal truncation of the neurokinin-2 receptor causes enhanced and sustained agonist-induced signaling- role of receptor phosphorylation in signal attenuation. *Journal of Biological Chemistry* **270**, 8944-8951
286. Alblas, J., vanEtten, I., and Moolenaar, W. H. (1996) Truncated, desensitization-defective neurokinin receptors mediate sustained MAP kinase activation, cell growth and transformation by a Ras-independent mechanism. *Embo Journal* **15**, 3351-3360

287. Eisinger, D. A., and Schulz, R. (2004) Extracellular signal-regulated kinase/mitogen-activated protein kinases block internalization of delta-opioid receptors. *Journal of Pharmacology and Experimental Therapeutics* **309**, 776-785
288. Lee, T. H., Woszczek, G., and Farooque, S. P. (2009) Leukotriene E-4: Perspective on the forgotten mediator. *Journal of Allergy and Clinical Immunology* **124**, 417-421
289. Steinke, J. W., Negri, J., Payne, S. C., and Borish, L. (2014) Biological effects of Leukotriene E4 on eosinophils. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, **91(3)**: 105-110
290. Kenakin, T. (1997) Differences between natural and recombinant G protein-coupled receptor systems with varying receptor G protein stoichiometry. *Trends in Pharmacological Sciences* **18**, 456-464
291. Paruchuri, S., Hallberg, B., Juhas, M., Larsson, C., and Sjolander, A. (2002) Leukotriene D-4 activates MAPK through a Ras-independent but PKC epsilon-dependent pathway in intestinal epithelial cells. *Journal of Cell Science* **115**, 1883-1893
292. Zhu, J., Qiu, Y.-S., Figueroa, D. J., Bandi, V., Galczenski, H., Hamada, K., Guntupalli, K. K., Evans, J. F., and Jeffery, P. K. (2005) Localization and upregulation of cysteinyl leukotriene-1 receptor in asthmatic bronchial mucosa. *American journal of respiratory cell and molecular biology* **33**, 531-540
293. Sousa, A. R., Parikh, A., Scadding, G., Corrigan, C. J., and Lee, T. H. (2002) Leukotriene-receptor expression on nasal mucosal inflammatory cells in aspirin-sensitive rhinosinusitis. *New England Journal of Medicine* **347**, 1493-1499
294. Corrigan, C., Mallett, K., Ying, S., Roberts, D., Parikh, A., Scadding, G., and Lee, T. (2005) Expression of the cysteinyl leukotriene receptors cysLT(1) and cysLT(2) in aspirin-sensitive and aspirin-tolerant chronic rhinosinusitis. *Journal of Allergy and Clinical Immunology* **115**, 316-322
295. Zhu, J., Bandi, V., Qiu, S., Figueroa, D. J., Evans, J. F., Barnes, N., Guntupalli, K. K., and Jeffery, P. K. (2012) Cysteinyl Leukotriene 1 Receptor Expression Associated With Bronchial Inflammation in Severe Exacerbations of COPD. *Chest* **142**, 347-357
296. Du, C., and Xie, X. (2012) G protein-coupled receptors as therapeutic targets for multiple sclerosis. *Cell Research* **22**, 1108-1128
297. Even-Ram, S., Uziely, B., Cohen, P., Grisaru-Granovsky, S., Maoz, M., Ginzburg, Y., Reich, R., Vlodavsky, I., and Bar-Shavit, R. (1998) Thrombin receptor overexpression in malignant and physiological invasion processes. *Nature Medicine* **4**, 909-914
298. Tantivejkul, K., Loberg, R. D., Mawocha, S. C., Day, L. L., St John, L., Pienta, B. A., Rubin, M. A., and Pienta, K. J. (2005) PAR1-mediated NF kappa B activation promotes survival of prostate cancer cells through a Bcl-xL-dependent mechanism. *Journal of Cellular Biochemistry* **96**, 641-652
299. Lee, Y. C., Lee, K. H., Lee, H. B., and Rhee, Y. K. (2001) Serum levels of interleukins (IL)-4, IL-5, IL-13, and interferon-gamma in acute asthma. *Journal of Asthma* **38**, 665-671
300. Ying, S., Humbert, M., Barkans, J., Corrigan, C. J., Pfister, R., Menz, G., Larche, M., Robinson, D. S., Durham, S. R., and Kay, A. B. (1997) Expression of IL-4 and IL-5 mRNA and protein product by CD4(+) and CD8(+) T cells, eosinophils, and mast cells in bronchial biopsies obtained from atopic and nonatopic (intrinsic) asthmatics. *Journal of Immunology* **158**, 3539-3544
301. Ying, S., Durham, S. R., Corrigan, C. J., Hamid, Q., and Kay, A. B. (1995) Phenotype pf cells expressing messenger-RNA for Th2-type (interleukin-4 and interleukin-5) and Th1-type (interleukin-2 and interferon-gamma) cytokines in bronchoalveolar lavage and bronchial biopsies from atopic asthmatic and normal asthmatic and normal control subjects. *American Journal of Respiratory Cell and Molecular Biology* **12**, 477-487
302. Ochi, H., De Jesus, N. H., Hsieh, F. H., Austen, K. F., and Boyce, J. A. (2000) IL-4 and-5 prime human mast cells for different profiles of IgE-dependent cytokine

production. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 10509-10513

303. Hsieh, F. H., Lam, B. K., Penrose, J. F., Austen, K. F., and Boyce, J. A. (2001) T helper cell type 2 cytokines coordinately regulate immunoglobulin E-dependent cysteinyl leukotriene production by human cord blood-derived mast cells: Profound induction of leukotriene C-4 synthase expression by interleukin 4. *Journal of Experimental Medicine* **193**, 123-133
304. Gessner, A., Mohrs, K., and Mohrs, M. (2005) Mast cells, basophils, and eosinophils acquire constitutive IL-4 and IL-13 transcripts during lineage differentiation that are sufficient for rapid cytokine production. *Journal of Immunology* **174**, 1063-1072
305. Knorr, B., Matz, J., Bernstein, J. A., Nguyen, H., Seidenberg, B. C., Reiss, T. F., Becker, A., and Pediat Montelukast Study, G. (1998) Montelukast for chronic asthma in 6- to 14-year-old children - A randomized, double-blind trial. *Jama-Journal of the American Medical Association* **279**, 1181-1186
306. Virchow, J. C., Mehta, A., Ljungblad, L., Mitfessel, H., and Grp, M. S. (2010) Add-on montelukast in inadequately controlled asthma patients in a 6-month open-label study: The MONtelukast In Chronic Asthma (MONICA) study. *Respiratory Medicine* **104**, 644-651
307. Vachier, I., Kumlin, M., Dahlen, S. E., Bousquet, J., Godard, P., and Chanez, P. (2003) High levels of urinary leukotriene E-4 excretion in steroid treated patients with severe asthma. *Respiratory Medicine* **97**, 1225-1229
308. Severien, C., Artlich, A., Jonas, S., and Becher, G. (2000) Urinary excretion of leukotriene E-4 and eosinophil protein X in children with atopic asthma. *European Respiratory Journal* **16**, 588-592
309. Biernacki, W. A., Kharitonov, S. A., Biernacka, H. M., and Barnes, P. J. (2005) Effect of montelukast on exhaled Leukotrienes and quality of life in asthmatic patients. *Chest* **128**, 1958-1963
310. Louis, R., Lau, L. C. K., Bron, A. O., Roldaan, A. C., Radermecker, M., and Djukanovic, R. (2000) The relationship between airways inflammation and asthma severity. *American Journal of Respiratory and Critical Care Medicine* **161**, 9-16
311. Asano, K., Shiomi, T., Hasegawa, N., Nakamura, H., Kudo, H., Matsuzaki, T., Hakuno, H., Fukunaga, K., Suzuki, Y., Kanazawa, M., and Yamaguchi, K. (2002) Leukotriene C-4 synthase gene A(-444)C polymorphism and clinical response to a CYS-LT1 antagonist, pranlukast, in Japanese patients with moderate asthma. *Pharmacogenetics* **12**, 565-570
312. Lima, J. J., Zhang, S., Grant, A., Shao, L. H., Tantisira, K. G., Allayee, H., Wang, L. W., Sylvester, J., Holbrook, J., Wise, R., Weiss, S. T., and Barnes, K. (2006) Influence of leukotriene pathway polymorphisms on response to montelukast in asthma. *American Journal of Respiratory and Critical Care Medicine* **173**, 379-385
313. Mougey, E. B., Feng, H., Castro, M., Irvin, C. G., and Lima, J. J. (2009) Absorption of montelukast is transporter mediated: a common variant of OATP2B1 is associated with reduced plasma concentrations and poor response. *Pharmacogenetics and Genomics* **19**, 129-138
314. Kim, K.-A., Lee, H.-M., Joo, H.-J., Park, I.-B., and Park, J.-Y. (2013) Effects of Polymorphisms of the SLCO2B1 Transporter Gene on the Pharmacokinetics of Montelukast in Humans. *Journal of Clinical Pharmacology* **53**, 1186-1193
315. Currie, G. P., Lima, J. J., Sylvester, J. E., Lee, D. K. C., Cockburn, W. J. R., and Lipworth, B. J. (2003) Leukotriene C-4 synthase polymorphisms and responsiveness to leukotriene antagonists in asthma. *British Journal of Clinical Pharmacology* **56**, 422-426
316. Ramires, R., Caiaffa, M. F., Tursi, A., Haeggstrom, J. Z., and Macchia, L. (2004) Novel inhibitory effect on 5-lipoxygenase activity by the anti-asthma drug montelukast. *Biochemical and Biophysical Research Communications* **324**, 815-821

317. Schmitt-Grohe, S., Eickmeier, O., Schubert, R., Bez, C., and Zielen, S. (2002) Anti-inflammatory effects of montelukast in mild cystic fibrosis. *Annals of Allergy Asthma & Immunology* **89**, 599-605
318. Stelmach, I., Korzeniewska, A., Stelmach, W., Majak, P., Grzelewski, T., and Jerzynska, J. (2005) Effects of montelukast treatment on clinical and inflammatory variables in patients with cystic fibrosis. *Annals of Allergy Asthma & Immunology* **95**, 372-380
319. Ichiyama, T., Hasegawa, S., Umeda, M., Terai, K., Matsubara, T., and Furukawa, S. (2003) Pranlukast inhibits NF-kappa B activation in human monocytes/macrophages and T cells. *Clinical and Experimental Allergy* **33**, 802-807
320. Ishinaga, H., Takeuchi, K., Kishioka, C., Suzuki, S., Basbaum, C., and Majima, Y. (2005) Pranlukast inhibits NF-kappa B activation and MUC2 gene expression in cultured human epithelial cells. *Pharmacology* **73**, 89-96
321. Fang, S. H., Yuan, Y. M., Peng, F., Li, C. T., Zhang, L. H., Lu, Y. B., Zhang, W. P., and Wei, E. Q. (2009) Pranlukast Attenuates Ischemia-like Injury in Endothelial Cells Via Inhibiting Reactive Oxygen Species Production and Nuclear Factor-kappa B Activation. *Journal of Cardiovascular Pharmacology* **53**, 77-85
322. Villaran, C., O'Neill, S. J., Helbling, A., van Noord, J. A., Lee, T. H., Chuchalin, A. G., Langley, S. J., Gunawardena, K. A., Suskovic, S., Laurenzi, M., Jasan, J., Menten, J., Leff, J. A., and Montelukast Salmeterol Exercise Study, G. (1999) Montelukast versus salmeterol in patients with asthma and exercise-induced bronchoconstriction. *Journal of Allergy and Clinical Immunology* **104**, 547-553
323. Fogel, R. B., Rosario, N., Aristizabal, G., Loeys, T., Noonan, G., Gaile, S., Smugar, S. S., and Polos, P. G. (2010) Effect of montelukast or salmeterol added to inhaled fluticasone on exercise-induced bronchoconstriction in children. *Annals of Allergy Asthma & Immunology* **104**, 511-517
324. Price, D. B., Swern, A., Tozzi, C. A., Philip, G., and Polos, P. (2006) Effect of montelukast on lung function in asthma patients with allergic rhinitis: analysis from the COMPACT trial. *Allergy* **61**, 737-742
325. Philip, G., Nayak, A. S., Berger, W. E., Leynadier, F., Vrijens, F., Dass, S. B., and Reiss, T. F. (2004) The effect of montelukast on rhinitis symptoms in patients with asthma and seasonal allergic rhinitis. *Current Medical Research and Opinion* **20**, 1549-1558
326. Borderias, L., Mincewicz, G., Paggiaro, P. L., Guilera, M., Kocevar, V. S., Taylor, S. D., and Badia, X. (2007) Asthma control in patients with asthma and allergic rhinitis receiving add-on montelukast therapy for 12 months: a retrospective observational study. *Current Medical Research and Opinion* **23**, 721-730
327. Keith, P. K., Koch, C., Djandji, M., Bouchard, J., Psaradellis, E., Sampalis, J. S., Schellenberg, R. R., and McIvor, R. A. (2009) Montelukast as add-on therapy with inhaled corticosteroids alone or inhaled corticosteroids and long-acting beta-2-agonists in the management of patients diagnosed with asthma and concurrent allergic rhinitis (the RADAR trial). *Canadian respiratory journal : journal of the Canadian Thoracic Society* **16**, 17A-31A
328. Peters-Golden, M., Swern, A., Bird, S. S., Hustad, C. M., Grant, E., and Edelman, J. M. (2006) Influence of body mass index on the response to asthma controller agents. *European Respiratory Journal* **27**, 495-503
329. Bisgaard, H., Zielen, S., Garcia-Garcia, M. L., Johnston, S. L., Gilles, L., Menten, J., Tozzi, C. A., and Polos, P. (2005) Montelukast reduces asthma exacerbations in 2-to 5-year-old children with intermittent asthma. *American Journal of Respiratory and Critical Care Medicine* **171**, 315-322
330. Christie, P. E., Schmitzschumann, M., Spur, B. W., and Lee, T. H. (1993) Airway responsiveness to leukotriene-C(4) (LTC(4)), leukotriene-E(4) (LTE(4)) and histamine in aspirin-sensitive asthmatic subjects. *European Respiratory Journal* **6**, 1468-1473

331. Higashi, N., Taniguchi, M., Mita, H., Yamaguchi, H., Ono, E., and Akiyama, K. (2012) Aspirin-Intolerant Asthma (AIA) Assessment Using the Urinary Biomarkers, Leukotriene E-4 (LTE4) and Prostaglandin D-2 (PGD(2)) Metabolites. *Allergology International* **61**, 393-403
332. Micheletto, C., Visconti, M., Tognella, S., Facchini, F. M., and Dal Negro, R. W. (2006) Aspirin induced asthma (AIA) with nasal polyps has the highest basal LTE4 excretion: a study vs AIA without polyps, mild topic asthma, and normal controls. *European annals of allergy and clinical immunology* **38**, 20-23
333. Gaber, F., Daham, K., Higashi, A., Higashi, N., Gulich, A., Delin, I., James, A., Skedinger, M., Gyllfors, P., Nord, M., Dahlen, S. E., Kumlin, M., and Dahlen, B. (2008) Increased levels of cysteinyl-leukotrienes in saliva, induced sputum, urine and blood from patients with aspirin-intolerant asthma. *Thorax* **63**, 1076-1082
334. Mastalerz, L., Gawlewicz-Mroczka, A., Cmiel, A., and Szczeklik, A. (2002) Protection against exercise-induced bronchoconstriction by montelukast in aspirin-sensitive and aspirin-tolerant patients with asthma. *Clinical and Experimental Allergy* **32**, 1360-1365
335. Foster, H. R., Fuerst, E., Lee, T. H., Cousins, D. J., and Woszczek, G. (2013) Characterisation of P2Y(12) Receptor Responsiveness to Cysteinyl Leukotrienes. *Plos One* **8(3)**: e58305

Chapter 9

Publications

9.1 Characterisation of $P2Y_{12}$ receptor responsiveness to cysteinyl leukotrienes

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Characterisation of $P2Y_{12}$ Receptor Responsiveness to Cysteinyl Leukotrienes

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Abstract

Leukotriene E_4 (LTE_4), the most stable of the cysteinyl leukotrienes (cysLTs), binds poorly to classical type 1 and 2 cysLT receptors although in asthmatic individuals it may potently induce bronchial constriction, airway hyperresponsiveness and inflammatory cell influx to the lung. A recent study has suggested that the purinergic receptor $P2Y_{12}$ is required for LTE_4 mediated pulmonary inflammation in a mouse model of asthma and signals in response to cysLTs. The aim of the study was to characterise the responsiveness of human $P2Y_{12}$ to cysteinyl leukotrienes. Models of human $CysLT_1$, $CysLT_2$ and $P2Y_{12}$ overexpressed in HEK293, CHO cells and human platelets were used and responsiveness to different agonists was measured using intracellular calcium, cAMP and β-arrestin recruitment assays. CysLTs induced concentration dependent calcium mobilisation in cells overexpressing $CysLT_1$ and $CysLT_2$ but failed to induce any calcium response in cells expressing $P2Y_{12}$ or $P2Y_{12}$+ $G\alpha_{16}$. In contrast, selective $P2Y_{12}$ agonists ADP and 2-MeS-ADP induced specific calcium flux in cells expressing $P2Y_{12}$+ $G\alpha_{16}$. Similarly, specific response to 2-MeS-ADP, but not to cysLTs was also observed in cells expressing $P2Y_{12}$ when intracellular cAMP and β-arrestin signalling were analysed. Platelets were used as a model of human primary cells expressing $P2Y_{12}$ to analyse potential signalling and cell activation through $P2Y_{12}$ receptor or receptor heterodimers but no specific LTE_4 responses were observed. These results show that LTE_4 as well as other cysLTs do not activate intracellular signalling acting through $P2Y_{12}$ and suggest that another LTE_4 specific receptor has yet to be identified.

Citation: Foster HR, Fuerst E, Lee TH, Cousins DJ, Woszczek G (2013) Characterisation of $P2Y_{12}$ Receptor Responsiveness to Cysteinyl Leukotrienes. PLoS ONE 8(3): e58305. doi:10.1371/journal.pone.0058305

Editor: Patricia T. Bozza, Fundação Oswaldo Cruz, Brazil

Received November 7, 2012; **Accepted** February 1, 2013; **Published** March 5, 2013

Funding: This work was supported by a grant from the Medical Research Council (G0900536) to Grzegorz Woszczek. The authors acknowledge support from the Department of Health via the National Institute for Health Research (NIHR) comprehensive Biomedical Research Centre award to Guy's & St Thomas' NHS Foundation Trust in partnership with King's College London and King's College Hospital NHS Foundation Trust. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Cysteinyl leukotrienes (cysLTs) (LTC_4, LTD_4, LTE_4) are an important class of proinflammatory lipid molecules that are thought to mediate many of the principal features of bronchial asthma such as bronchial constriction, airway hyperresponsiveness and leukocyte trafficking. They are synthesized *in vivo* by immunocompetent cells such as mast cells, eosinophils, basophils and monocytes/macrophages [1]. Upon cell activation, intracellular phospholipase A_2 releases arachidonic acid from membrane phospholipids. 5-lipoxygenase subsequently converts arachidonic acid to the unstable intermediate LTA_4, which is then conjugated to reduced glutathione by leukotriene C_4 synthase to form LTC_4. After transport to extracellular space, LTC_4 is converted to LTD_4 and then to the terminal product LTE_4, the most stable and the most abundant cysLT in biological fluids.

The biological actions of cysLTs are mediated by two currently identified G-protein coupled receptors (GPCR): CysLT type 1 receptor ($CysLT_1$) and type 2 receptor ($CysLT_2$). They differ in binding affinities for different cysLTs. $CysLT_1$ is recognised as a high-affinity receptor for LTD_4, whereas $CysLT_2$ binds LTC_4 and LTD_4 with similar affinity [2,3,4,5]. LTE_4 has long been believed to be the final and least active metabolite of cysLTs, with low affinity for binding to the classical receptors and lowest functional agonistic potency in comparison to LTC_4 and LTD_4 [6].

Inhaled LTD_4 has been shown to elevate the numbers of sputum eosinophils in subjects with asthma [7]. However, it was LTE_4 that was shown to be the most potent cysLT in eliciting influx of inflammatory cells such as eosinophils and basophils into bronchial mucosa of asthmatic subjects [8,9]. These preferential agonist functions of LTE_4 were not explained by pharmacological properties of $CysLT_1$ or $CysLT_2$. Similarly, there is still no explanation for the comparable potency of LTE_4 in comparison to LTC_4 and LTD_4 in eliciting a dermal wheal and flare reaction in human skin [10] and for equally effective contraction of human bronchi *in vitro* by all cysLTs [11]. All of the above data strongly suggests the existence of one or more specific LTE_4 receptors that have not been identified to date. The potential existence of such a receptor has been recently demonstrated [12]. In a knock-out murine model, vascular permeability induced by intradermal injection of LTE_4 in mice lacking both $CysLT_1$ and $CysLT_2$ exceeded the response to LTC_4 and LTD_4, suggesting the presence of another cysLT receptor that responds preferentially to LTE_4. LTE_4 was 64-fold more potent in $CysLT_1$/$CysLT_2$ double deficient mice than in wild type mice, revealing an inhibitory negative regulation of the novel LTE_4 receptor by the two known receptors. It was also found that the pre-treatment of

double deficient mice with a selective $CysLT_1$ antagonist did not inhibit, but increased even further the permeability response to all cysLTs.

Interestingly, recent *in silico* modelling and *in vitro* studies suggested that LTE_4 may be a ligand for an ADP receptor, $P2Y_{12}$ when heterologously expressed as a fusion protein with human $G\alpha_{16}$ [13]. Further evidence for the interaction of LTE_4 with the purinergic receptor $P2Y_{12}$ was provided by Paruchuri *et al.* who showed that $P2Y_{12}$ was required for LTE_4-mediated pulmonary inflammation [14]. Mice lacking the classical cysLT receptors maintained LTE_4-induced eosinophilia, goblet cell metaplasia and IL-13 expression in response to low-dose of aerosolized allergen but $P2Y_{12}$ knock-out and platelet-depleted mice (a cell type highly expressing $P2Y_{12}$) showed a substantial loss in those functions. Although direct binding of labelled LTE_4 to $P2Y_{12}$ could not be demonstrated, data from cells overexpressing $P2Y_{12}$ indicated that the presence of $P2Y_{12}$ is required for signalling and activation by LTE_4.

Human $P2Y_{12}$ has been cloned and characterised with ADP as its natural agonist [15]. It has been shown to couple to $G\alpha_i$ and signal in response to ADP by inhibiting adenylate cyclase activity and cAMP generation in human platelets and when heterologously expressed [16]. To address the question whether LTE_4 is also a direct agonist for human $P2Y_{12}$ or activates $P2Y_{12}$ signalling through GPCR heterodimer interactions we characterised responsiveness to cysLTs in recombinant models of cell overexpressing $P2Y_{12}$ and in platelets, primary human cells constitutively expressing $P2Y_{12}$.

Materials and Methods

Reagents

Leukotrienes (LTC_4, LTD_4 and LTE_4) were purchased from Cayman Chemical (Ann Arbor, Mich). ADP, 2-methylthioadenosine-5′-diphosphate (2MeS-ADP), prostaglandin E_1 (PGE_1), isoproterenol, forskolin, 3-Isobutyl-1-methylxanthine (IBMX) and calcium ionophore (A23187) were purchased from Sigma-Aldrich (Dorset, UK).

Cell Culture

HEK293 cells were cultured in high glucose (4500 mg/L) DMEM supplemented with 2 mmol/L glutamine, 10% fetal bovine serum and Penicillin/Streptomycin (50 units/ml/50 μg/ml) (all Life Technologies, UK) in a humidified 5% CO_2 37°C incubator. Cells were passaged every 3–4 days replacing all medium with fresh cell culture medium.

Transient Transfection of HEK293 Cells

HEK293 cells cultured to above 60% confluence were transiently transfected with a mixture of Lipofectamine 2000 (Life Technologies) and the following plasmids as indicated: pcDNA3.1-human $CysLT_1$, pcDNA3.1-human $CysLT_2$, pcDNA3.1-human $P2Y_{12}$, pcDNA3.1-3xHA human $P2Y_{12}$, pcDNA3.1-human $G\alpha_{16}$, pcDNA3.1-3xHA human ADRB2 (all the Missouri S&T cDNA Resource Center, Rolla, Mo) and pCMV6-Kan/Neo- mouse $P2Y_{12}$ (Origene Technologies) in serum-free medium (Opti-MEM, Life Technologies) according to manufacturer's protocol. After incubation the transfection medium was removed and HEK293 cells were cultured for 36 hours in standard culture medium at 37°C in a humidified 5% CO_2 incubator.

Preparation of Platelet-rich Plasma

The study was approved by the Research Ethics Committee of Guy's Hospital. Blood was collected over citrate-dextrose solution (ACD, 6:1) from patients who had provided written informed consent prior to any procedure. Platelet-rich plasma (PRP) was prepared by centrifugation at 150 g for 15 minutes at room temperature. PRP was centrifuged for a further 5 minutes to reduce erythrocyte contamination.

Calcium Mobilisation Assay

Calcium mobilisation assays were conducted using FLIPR calcium 4 assay kit (Molecular Devices, Sunnydale, CA) as described previously [17,18]. HEK293 cells (1.5×10^5/well) were plated into poly-D-lysine coated 96 well plates in RPMI 1640 supplemented with 10 mmol/L HEPES. After a 5-hour incubation, cells were incubated for 1 hour with FLIPR loading buffer prior to addition of ligand and fluorescent intensity was measured at 37°C using a Flexstation 3 (Molecular Devices). Controls included medium control with ethanol for leukotriene stimulations.

PRP was washed in modified Tyrode's buffer (pH 6.2, 150 mol/L NaCl, 3 mmol/L KCl, 5 mmol/L glucose, 1 mmol/L $MgCl_2$, 10 mmol/L HEPES, 0.1% BSA) supplemented with 0.5 μmol/L PGE_1. Platelets (2×10^6/well) were plated into 96-well plates in modified Tyrode's buffer supplemented with 1.26 mmol/L $CaCl_2$ and incubated for 30 minutes with FLIPR loading buffer supplemented with 2.5 mmol/L probenecid and fluorescence was measured using a Flexstation 3. Results were analysed with SoftMax Pro Software (Molecular Devices).

Analysis of Receptor Surface Expression

Washed transfected HEK293 cells were stained with an Alexa Fluor 488 conjugated anti-HA monoclonal antibody (Clone 16B12, Covance, Ca) that recognises the HA epitope that is N-terminally located on the receptors of interest. Analysis was performed on a FACScalibur with CellQuest Pro software (BD Biosciences).

cAMP Accumulation Assay

Intracellular cAMP accumulation was analysed in HEK293 cells using the Cyclic AMP assay kit (Meso Scale Discovery, Gaithersburg, MD, USA) following manufacturer's protocols. HEK293 cells with added IBMX (1 μmol/L) were plated on anti-cAMP coated MULTI-ARRAY 96-well small spot plates (MSD), stimulated with forskolin and agonists for 15 minutes as indicated, lysed and run on the ImageSector 6000 (Meso Scale Discovery). Results were analysed using MSD workbench software.

Intracellular cAMP accumulation was analysed in PRP using the HitHunter® cAMP XS+ assay kit (DiscoverX, UK) following manufacturer's protocols. PRP was centrifuged at 1000 g for 10 minutes and washed three times with pre-chilled DPBS supplemented with 2 mmol/L EDTA. Platelets (5×10^6/well) suspended in DPBS supplemented with 2 mmol/L EDTA and 1 μmol/L IBMX, were plated on a 96-well plate, stimulated with forskolin and other agonists for 20 minutes as indicated and incubated with detection reagents. Luminescent signal was measured 4 hours after lysis using a Flexstation 3. Results were analysed with SoftMax Pro Software.

β-arrestin Recruitment Assay

Analysis of β-arrestin recruitment was conducted using a Pathhunter eXpress β-arrestin kit (DiscoverX) following manufacturer's protocols. In this system, the GPCR and β-arrestin are fused to two fragments of β-galactosidase and the interaction of the two proteins results in an enzymatic reaction. In brief, CHO cells stably transfected with the C-terminally modified human

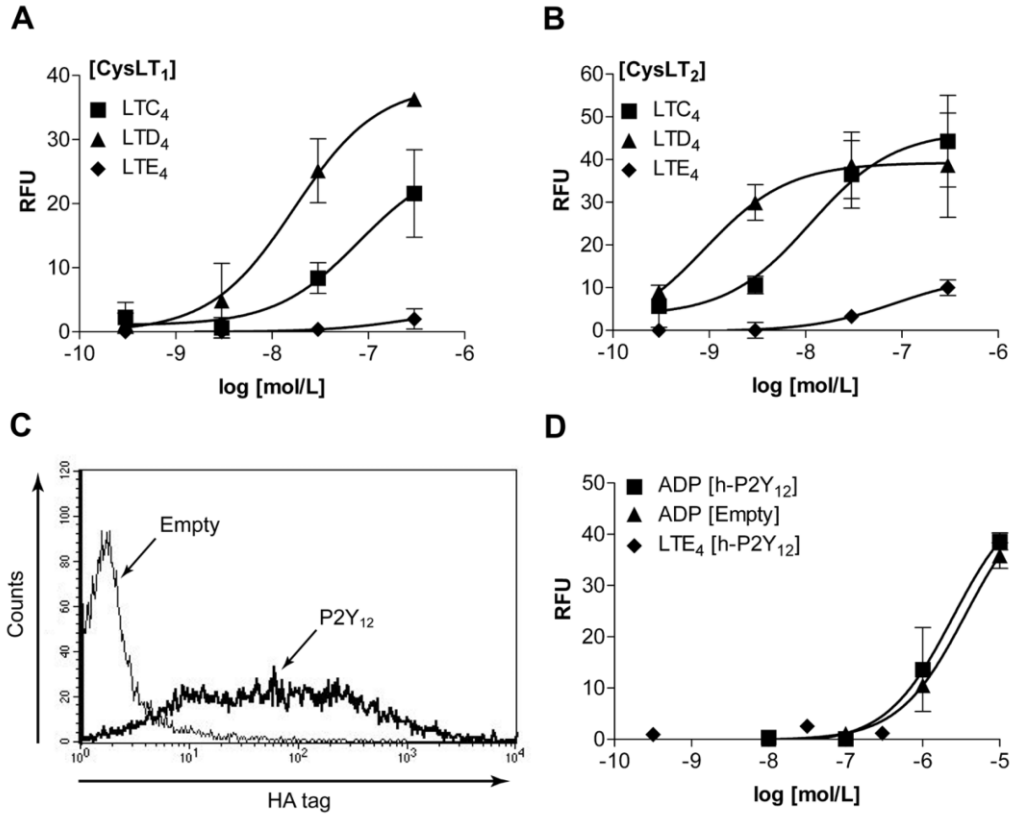

Figure 1. Effect of LTE_4 on calcium mobilisation in h-$P2Y_{12}$ overexpression model. HEK293 cells were transiently transfected with indicated vectors and intracellular responses recorded. (A) [h-$CysLT_1$] and (B) [h-$CysLT_2$] transfectants stimulated with indicated concentrations of LTC_4, LTD_4 and LTE_4. N = 6. (C) Flow cytometry analysis of h-$P2Y_{12}$ protein surface expression in [h-$P2Y_{12}$] and [Empty] transfectants using an anti-HA antibody, representative of three experiments. (D) [h-$P2Y_{12}$] and [Empty] transfectants stimulated with ADP or LTE_4, N = 9. Results (A), (B) and (D) represented as peak intracellular calcium response, relative fluorescence units (RFU), mean ± S.E.M.
doi:10.1371/journal.pone.0058305.g001

(mouse) $P2Y_{12}$ and with the β-arrestin, N-terminally tagged with deletion mutant of β-galactosidase, were seeded on 96-well plates in OCC medium for a 48 hour recovery period at 37°C. Cells were stimulated for 90 minutes at 37°C as indicated and then incubated with detection reagents for a further 90 minutes at room temperature. Luminescent signal, which is directly related to the recruitment of β-arrestin to $P2Y_{12}$ in the assay, was measured using a Flexstation 3.

Analysis of Platelet Activation by Flow Cytometry

Whole peripheral blood drawn over ACD (6:1) was stimulated immediately after collection for 10 minutes at room temperature. 5 µl of blood was directly stained with monoclonal antibodies against CD61 (APC, clone VI-PL2) and CD62P (PE, clone Psel.KO2.3) or appropriate isotype controls (all eBiosciences). Cells gated in the platelet population were analysed for platelet activation using a FACScalibur with CellQuest Pro software (BD Biosciences).

CCL5/RANTES ELISA

PRP supplemented with 0.5 µmol/L PGE_1 was centrifuged at 1000 g for 10 minutes and washed three times with pre-warmed modified Tyrode's buffer supplemented with 0.5 µmol/L PGE_1. PRP was stimulated for 15 minutes with indicated agonists and CCL5 was measured in supernatants using a CCL5/RANTES duo set kit (R&D Systems, UK) following manufacturer's protocol. Optical density was recorded on Anthos htIII (Anthos Labtech) using Stingray (DazDaq) software. Measurements at 450 nm were corrected by measurement at 578 nm and concentrations of RANTES/CCL5 were generated from the standard curve.

Statistical Analysis

Data were analysed by means of one- or two- way ANOVA using GraphPad Prism software (GraphPad, La Jolla, Ca). Differences were considered significant at a p-value of less than 0.05.

Results

CysLT-mediated Calcium Mobilisation in Models of Transiently Expressed $CysLT_1$, $CysLT_2$ and $P2Y_{12}$

To ascertain whether LTE_4 could mediate signal transduction through the $P2Y_{12}$ receptor, a model of heterologous receptor expression was established in HEK293 cells. These cells do not natively express any known classical cysLT receptors or $P2Y_{12}$ and when unmodified they do not respond to cysLT stimulation. To validate this model, constructs expressing human (h)-$CysLT_1$ and h-$CysLT_2$ were transiently transfected into separate HEK293 populations as previously described [5]. The transfectants were stimulated with exogenous LTC_4, LTD_4 and LTE_4 and their intracellular calcium responses were measured using FLIPR Calcium 4 assay kit and a FlexStation 3, showing similar receptor potency as reported previously, with LTE_4 having the lowest potency for calcium mobilisation in comparison to LTC_4 and LTD_4. (Fig. 1A–B) [19]. Constructs expressing h-$P2Y_{12}$ with N-terminal 3xHA tag were then transiently transfected into HEK293 cells and surface expression was verified by flow cytometry (Fig. 1C). On stimulation with exogenous cysLTs or ADP, the known natural ligand for h-$P2Y_{12}$, no difference was found in intracellular calcium flux responses between the h-$P2Y_{12}$ transfectants and controls transfected with empty vectors (Fig. 1D), although calcium response to ADP was observed reflecting constitutive expression of other purinergic receptors and showing that h-$P2Y_{12}$ signal transduction does not occur through calcium mobilisation in this model.

LTE_4-mediated Calcium Mobilisation in $G\alpha_{16}$ co-transfection Models

Co-transfections of G-protein coupled receptors (GPCRs) and the fusion protein, $G\alpha_{16}$, have been reported previously to directly activate phospholipase C and calcium signalling [20]. To demonstrate the effectiveness of this approach in our recombinant model we overexpressed human β_2 adrenergic (h-$ADR\beta_2$) receptor and stimulated with isoproterenol. A construct encoding h-$ADR\beta_2$ with N-terminal 3xHA tag was transiently co-transfected with a construct containing h-$G\alpha_{16}$ into HEK293 cells and surface expression of the receptor was verified by flow cytometry (Fig. 2A). On stimulation with exogenous isoproterenol, h-$ADR\beta_2$ transfectants overexpressing h-$G\alpha_{16}$ were able to produce a statistically significant ($p = 0.0003$; 2-way ANOVA) increase in calcium flux compared to h-$ADR\beta_2$ transfectants showing that h-$G\alpha_{16}$ has the potential to modulate GPCR signal transduction in our HEK293 heterologous expression model (Fig. 2B).

Constructs containing tagged h-$P2Y_{12}$ were then co-transfected with h-$G\alpha_{16}$ and receptor surface expression was confirmed by flow cytometry (Fig. 2A). These transfectants were also able to show statistically significant increases in calcium responses to ADP ($p = 0.022$; 2-way ANOVA) and its more stable derivative, 2-MeS-ADP ($p = 0.019$; 2-way ANOVA), compared to control transfectants (fig. 2C–D). Negligible increases in calcium flux were observed upon LTE_4 stimulation of the h-$P2Y_{12}$+ h-$G_{\alpha 16}$ transfectants (Fig. 2C–D) and upon stimulation with LTC_4 and LTD_4 (data not shown).

$P2Y_{12}$-induced Intracellular cAMP Signalling

Human $P2Y_{12}$ has been shown to signal physiologically through $G\alpha_i$ and by inhibition of intracellular cAMP generation. To analyse this potential signalling pathway, h-$P2Y_{12}$ transfectants were stimulated with forskolin to activate adenylyl cyclase and to increase cAMP levels, together with ADP or cysLTs and intracellular cAMP was measured using a competitive immunoassay. ADP induced a significant, concentration dependent inhibition of forskolin induced cAMP in h-$P2Y_{12}$ transfectants, while LTE_4 treatment showed no statistically significant difference in cAMP accumulation over a range of 0.3–300 nmol/L concentrations, from the empty vector transfectants (Fig. 3A). This confirms that h-$P2Y_{12}$ does not signal directly through the coupling of either to $G\alpha_q$ or $G\alpha_i$ upon LTE_4 stimulation. We could also exclude $G\alpha_s$ signalling in our model as no change in cAMP levels was observed when h-$P2Y_{12}$ transfected cells were stimulated with ADP or cysLTs alone (not shown).

$P2Y_{12}$-induced β-arrestin Signalling

Recruitment of β-arrestin is another activation event that can be analysed as a measure of GPCR activation. Until recently, an agonist's efficacy for β-arrestin recruitment was believed to be proportional to its efficacy for G-protein activities. However, it has been demonstrated that "biased ligands" can selectively activate β-arrestin function and elicit specific biological effects [21]. To address the question of β-arrestin specific signalling induced by LTE_4, C-terminally modified h-$P2Y_{12}$ stably transfected into CHO cells with β-arrestin N-terminally tagged with a deletion mutant of β-galactosidase were stimulated with either 2-MeS-ADP or cysLTs and processed according to manufacturer's protocol (Fig. 3B). Stimulation with 2-MeS-ADP induced a concentration dependent luminescent signal relating to the recruitment of β-arrestin to the h-$P2Y_{12}$ receptor. Stimulation with LTE_4 and other cysLTs showed no significant increase in signal suggesting a lack of β-arrestin pathway activation by leukotrienes. Collectively these observations show that cysLTs do not induce G-protein dependent or independent signalling pathways directly through h-$P2Y_{12}$ indicating that h-$P2Y_{12}$ is not a cysLT receptor.

LTE_4-mediated Signalling through Mouse $P2Y_{12}$

The ability of LTE_4 to mediate pulmonary inflammation has been shown to be dependent on $P2Y_{12}$ expression in mouse models, as indicated by Paruchuri *et al.* [14]. Strong evidence shows that on removal of the $P2Y_{12}$ receptor, either by knock down or by platelet depletion, the influx of inflammatory cells to the mouse lung can be significantly diminished. Although these findings are contradictory to our results, the lack of LTE_4 mediated signalling in our recombinant model could be due to species differences as mouse (m)-$P2Y_{12}$ shares only 89% homology to its human derivative [22]. To test this possibility a construct encoding m-$P2Y_{12}$ were transiently transfected into HEK293 cells and stimulated with exogenous 2-MeS-ADP, LTC_4, LTD_4 or LTE_4. Although a robust calcium flux was detected in response to 2-MeS-ADP stimulation, it did not differ in comparison to empty vector control transfectants and no response, similar to that of the human $P2Y_{12}$ (Fig. 1D), was recorded for cysLT stimulation (data not shown). Co-transfections of m-$P2Y_{12}$ and $G\alpha_{16}$ were then employed to direct any signal transduction to activate calcium mobilisation. 2-MeS-ADP stimulation of these co-transfectants was able to induce a statistically significant increase in calcium mobilisation compared to the control transfectants (Fig. 4A) indicating that h-$G\alpha_{16}$ is sufficiently able to direct the signalling pathway of m-$P2Y_{12}$. No specific calcium flux was observed on stimulation of these co-transfectants with LTE_4 (Fig. 4A).

To determine whether LTE_4 could activate m-$P2Y_{12}$ via the recruitment of β-arrestin, stable CHO cell transfectants of C-terminally modified m-$P2Y_{12}$ and N-terminally tagged β-arrestin were stimulated with either 2-MeS-ADP or cysLT. Although stimulation with 2-MeS-ADP induced a dose-dependent luminescent signal, LTE_4 stimulation produced negligible effects (Fig. 4B). These results show that m-$P2Y_{12}$ signalling responses towards cysLT stimulation are similar to that of h-$P2Y_{12}$.

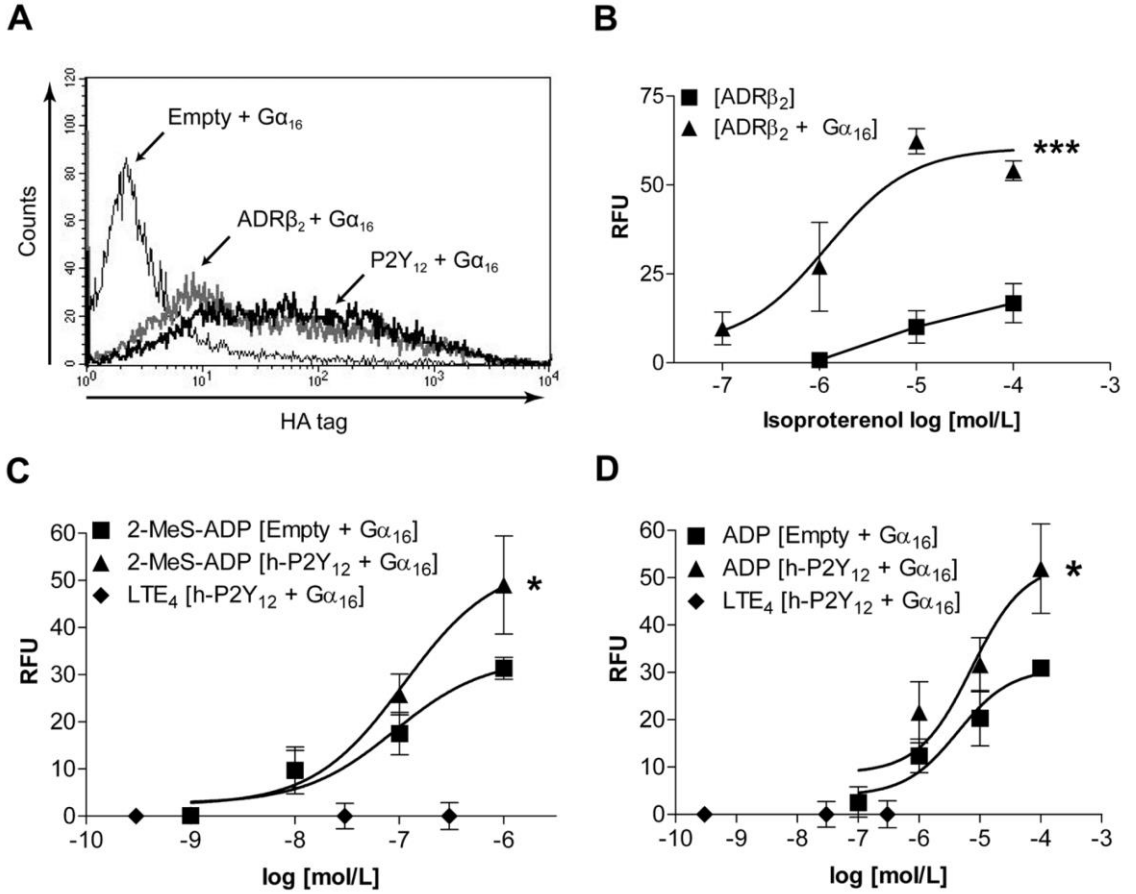

Figure 2. Effect of LTE_4 on HEK293 cells co-transfected with h-$P2Y_{12}$ and $G_{\alpha 16}$. HEK293 cells were transiently transfected with vectors indicated and intracellular responses recorded. (A) Flow cytometry analysis of $ADR\beta_2$ and h-$P2Y_{12}$ protein surface expression in [$ADR\beta_2$+ $G_{\alpha 16}$] and [h$P2Y_{12}$+ $G_{\alpha 16}$] transfectants respectively, representative of three experiments. $ADR\beta_2$ and h-$P2Y_{12}$ were detected using an anti-HA antibody, representative of three experiments. (B) [$ADR\beta_2$] and [$ADR\beta_2$+ $G_{\alpha 16}$] transfectants stimulated with isoproterenol, N = 6, 2-way ANOVA p = 0.0003. [h-$P2Y_{12}$+ $G_{\alpha 16}$] and [Empty+$G_{\alpha 16}$] transfectants stimulated with LTE_4 and either (C) 2-MeS-ADP or (D) ADP, N = 9. 2-way ANOVA between (C) 2-MeS-ADP responses p = 0.0190 and (D) ADP responses p = 0.0220. Results (B), (C) and (D) presented as peak intracellular calcium response mean ± S.E.M.
doi:10.1371/journal.pone.0058305.g002

LTE_4-induced Signalling and Cell Activation in Human Platelets

Physiologically, the signalling capability of $P2Y_{12}$ is highly modulated by another ADP purinergic receptor, $P2Y_1$ [23]. Signal modulation, whether this is through heterodimerisation or reciprocal cross-talk, allows the potentiation of signalling responses from both $P2Y_{12}$ and $P2Y_1$. To determine whether the importance of $P2Y_{12}$ in LTE_4 mediated pulmonary inflammation *in vivo* could be due to such interactions or heterodimerisation of GPCRs, isolated human platelets, one of very few cell types that highly expresses $P2Y_{12}$, were stimulated with 2-MeS-ADP and LTE_4 and their intracellular signalling responses were analysed. A robust calcium mobilisation in a dose dependent manner and a statistically significant inhibition of cAMP were generated by 2-MeS-ADP stimulation (Fig. 5A–B) indicating that platelets isolated from whole blood were functionally intact and are responsive to $P2Y_{12}$ agonists. LTE_4 stimulation was unable to generate any calcium mobilisation and no significant inhibition of cAMP was observed (Fig. 5A–B) which is in agreement with the recombinant model data (Fig. 1D and 3A).

Activated platelets enter into an aggregation cascade where adhesion molecules are upregulated on the cell surface and range of stored mediators are released to enhance this process. To address the question whether cysLTs are able to activate platelets through $P2Y_{12}$ or physiologically expressed receptor heterodimers, platelet activation measured by expression of P-selectin (CD62P) and release of stored chemokine CCL5 (RANTES) were analysed. Whole blood was stimulated with either ADP, 2-MeS-ADP or cysLTs and CD62P expression was measured by flow cytometry on the $CD61^+$ population of human platelets (Fig. 5C). Whereas a robust upregulation of CD62P was observed after ADP or 2-MeS-ADP stimulation, no specific response to LTE_4 (Fig. 5C–D) or other cysLTs (data not shown) was observed. Similarly, as human platelets stimulated with calcium ionophore or ADP released increased amounts of CCL5, no such a response was

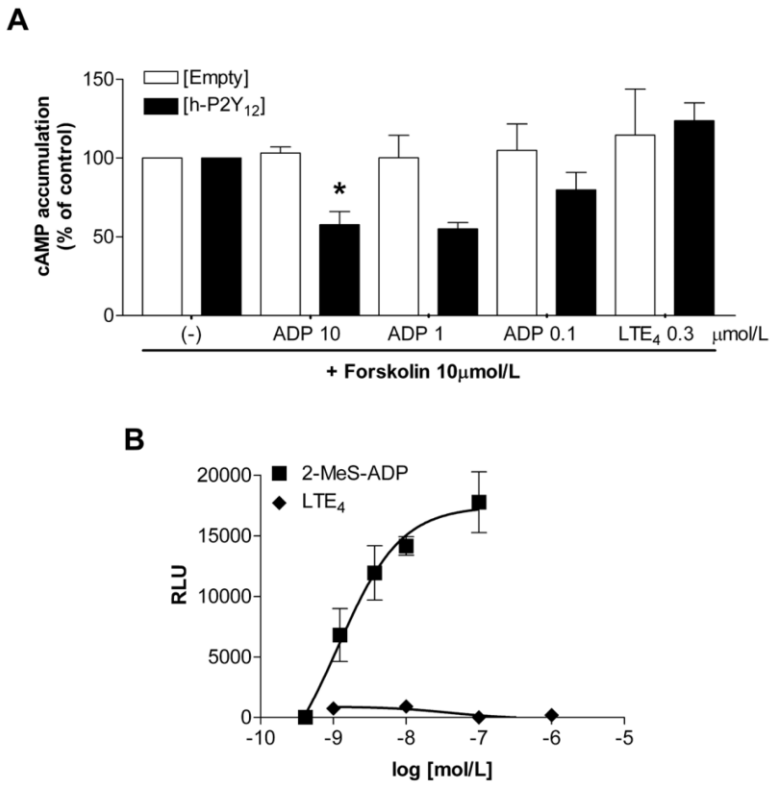

Figure 3. Effect of LTE_4 stimulation on cAMP and β-arrestin signalling pathways. Intracellular cAMP concentrations and β-arrestin recruitment was analysed in models of transiently transfected HEK293 or stably modified CHO cells, respectively. (A) [h-$P2Y_{12}$] and [Empty] transfectants were stimulated with forskolin and either ADP or LTE_4, N = 6, data expressed as % of forskolin stimulated control. (B) CHO cells expressing h-$P2Y_{12}$ and β-arrestin were stimulated with either 2-MeS-ADP or LTE_4, N = 9, expressed as relative luminescence units (RLU). Data represented as mean ± S.E.M. Two-way ANOVA with Bonferroni post-hoc test, *$p<0.05$.
doi:10.1371/journal.pone.0058305.g003

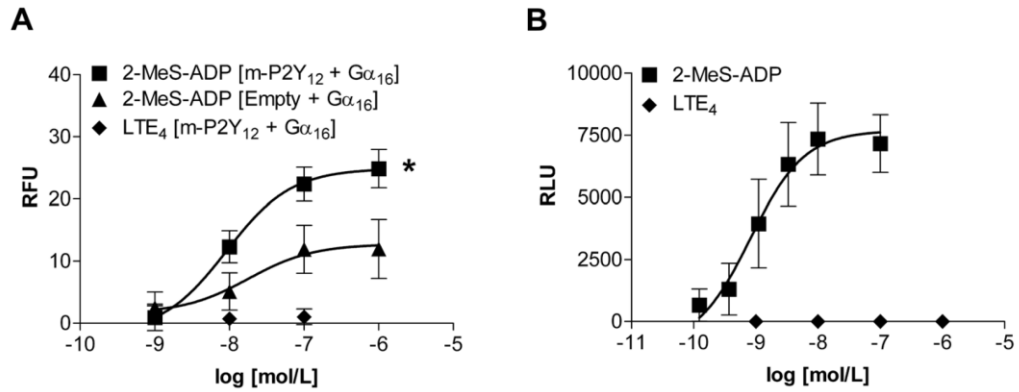

Figure 4. Effect of LTE_4 stimulation on calcium and β-arrestin signalling pathways in mouse $P2Y_{12}$ transfectants. HEK293 cells were transiently transfected with indicated vectors and intracellular calcium responses recorded. (A) [m-$P2Y_{12}$+ $G_{\alpha 16}$] and [Empty+$G_{\alpha 16}$] transfectants were stimulated with LTE_4 and 2-MeS-ADP, N = 9, 2-way ANOVA between 2-MeS-ADP responses $p = 0.0101$. (B) CHO cells stably expressing m-$P2Y_{12}$ and β-arrestin were stimulated with either 2-MeS-ADP or LTE_4, N = 9. Data presented as mean ± S.E.M.
doi:10.1371/journal.pone.0058305.g004

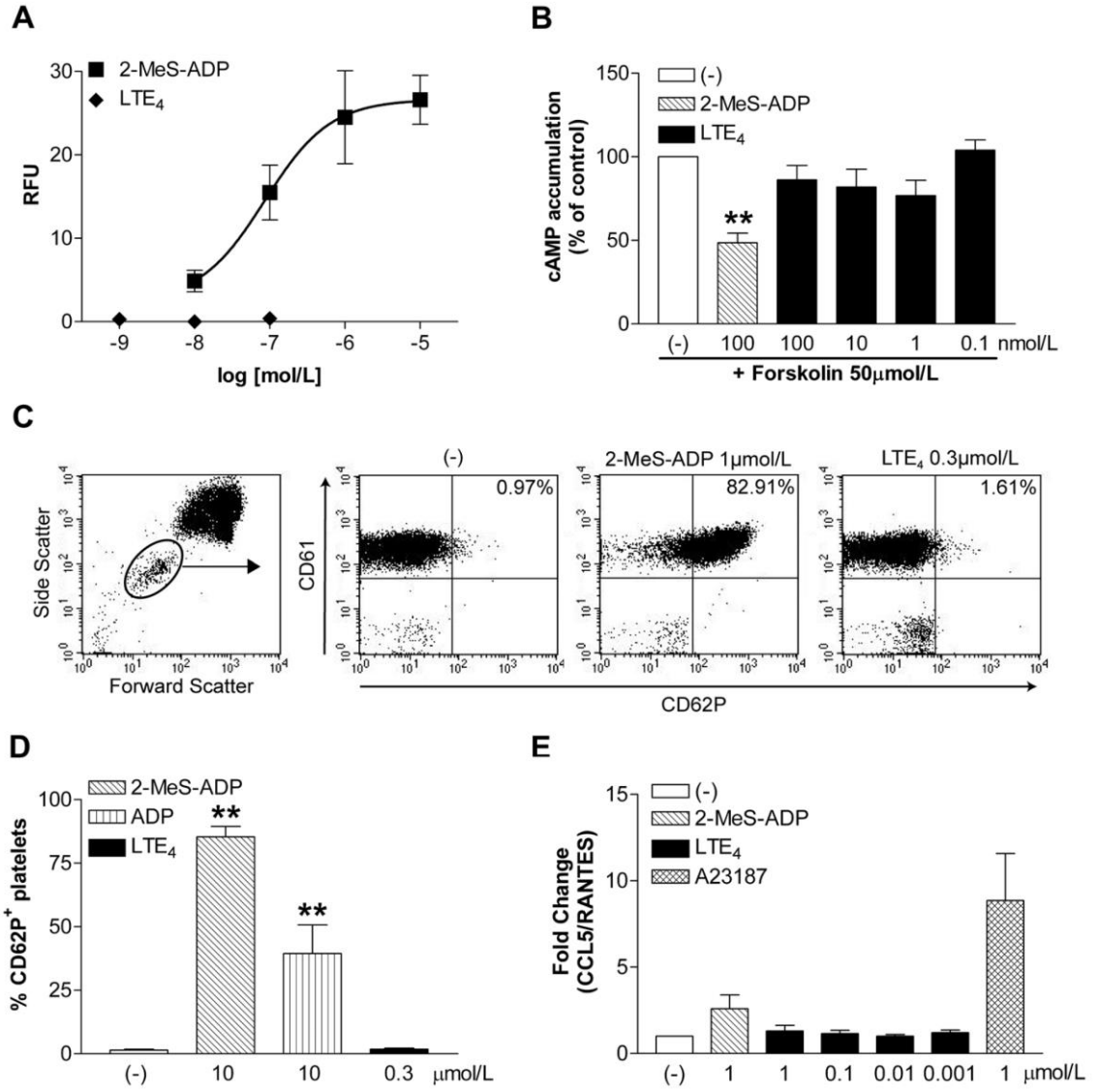

Figure 5. The effect of LTE$_4$ stimulation on human platelets. Isolated human platelets were (A) stimulated with 2-MeS-ADP and LTE$_4$ and peak intracellular calcium mobilisation responses were recorded, data from 3 donors run in duplicate, (B) stimulated with forskolin and either LTE$_4$ or 2-MeS-ADP for 15 minutes in the presence of IBMX, data from 3–6 donors run in triplicate, expressed as % of forskolin stimulated control. (C) Whole blood stimulated with either 2-MeS-ADP or LTE$_4$ for 10 minutes was stained with monoclonal antibodies for CD61 and CD62P. Flow cytometry analysis of platelet activation was performed on gated platelet population, representative of three experiments. (D) Flow cytometry analysis of platelet activation, data presented as mean ± S.E.M of 3 experiments with different donors. (E) Isolated human platelets were stimulated with either LTE$_4$, 2-MeS-ADP or calcium ionophore, supernatants collected and CCL5 concentrations measured using ELISA. Data presented as mean ± S.E.M of 3 experiments with different donors run in duplicate. One-way ANOVA performed **$p<0.01$.
doi:10.1371/journal.pone.0058305.g005

identified upon stimulation with LTE$_4$ (Fig. 5E) or other cysLTs (data not shown).

Discussion

This study highlights the complexities in leukotriene receptor biology and leukotriene signalling pathways involved in the immune response in chronic inflammatory diseases, such as atherosclerosis, asthma and rhinosinusitis. Ever since the elucidation and cloning of the two human cysLT receptors, CysLT$_1$ and CysLT$_2$, LTE$_4$ has become the forgotten mediator in cysLT biology [6]. Its apparent weak efficacy in recombinant systems, poor binding affinities compared to LTC$_4$ and LTD$_4$, and the availability of selective CysLT$_1$ antagonists, sidelined LTE$_4$ as an

important target for basic and clinical research. Recent studies have re-examined the role of LTE_4 in pulmonary inflammation in relation to $P2Y_{12}$ (a receptor proposed as a putative LTE_4 receptor) [13]. Low dose OVA-induced allergic lung inflammation murine models lacking $P2Y_{12}$ functionality either by knock down, platelet depletion or receptor antagonism, exhibited a substantial reduction in LTE_4 mediated pulmonary inflammation, a phenomenon not observed in $CysLT_1/CysLT_2$ double knock out mice [14]. We undertook this study to determine whether these observations were specifically related to direct $P2Y_{12}$-LTE_4 interactions, therefore determining whether $P2Y_{12}$ was in fact a cysteinyl leukotriene receptor.

Firstly, we established the heterologous expression model in which the recombinant receptors of interest could be transiently overexpressed in HEK293 cells. The best understood cysteinyl leukotriene receptor signalling pathway couples to $G\alpha_q$ thus functional validation of the expression model was carried out by analysing intracellular calcium mobilisation. The pattern of ligand efficacies in both $CysLT_1$ and $CysLT_2$ transfectants matched those observed in the literature (LTD_4 being the most potent agonist and LTE_4 being the weakest) (Fig. 1) and with $CysLT_1$ responses being sensitive to the specific $CysLT_1$ antagonist, MK-571 (data not shown), therefore validating the expression model. Unsurprisingly, calcium mobilisation was not induced by cysteinyl leukotriene stimulation of $P2Y_{12}$ transfectants, as the purinergic receptor has been well characterised as a GPCR that mainly activates $G\alpha_i$ signalling pathways, affecting intracellular cAMP levels. However, the lack of LTE_4 calcium mobilisation in $P2Y_{12}$ transfectants co-expressing the $G\alpha_{16}$ protein was in stark contrast with the initial study by Nonaka *et al.* identifying LTE_4 as a surrogate ligand for $P2Y_{12}$ [13]. They showed that LTE_4, but not LTC_4 or LTD_4, was able to induce intracellular calcium flux in CHO cells stably overexpressing both h-$P2Y_{12}$ and $G\alpha_{16}$. As different platforms for the recombinant overexpression model were utilised, as well as different transfection techniques, this could be a reason why different signalling responses were observed. Further confirmation of our observations was shown by the negligible effect of LTE_4 and other cysteinyl leukotrienes on cAMP accumulation and β-arrestin recruitment (Fig. 3), two intracellular signalling pathways being potently activated in the same assays by known $P2Y_{12}$ agonists, ADP and 2-MeS-ADP. This lack of LTE_4 induced signalling was mimicked in transfectants containing the mouse version of the $P2Y_{12}$ receptor (Fig. 4) suggesting that the deficiency in signalling responses was not merely a human phenomenon and was independent of species variation. As no direct $P2Y_{12}$ signalling upon cysLTs stimulation was observed in any of our recombinant experiments, we decided to address another possibility that LTE_4 activates cells through another GPCR forming a heterodimer with $P2Y_{12}$ *in vivo*. Platelets are one of very few cell types that functionally express $P2Y_{12}$ and platelet depletion potently inhibited LTE_4 mediated pulmonary inflammation (9) so we used human platelets to verify whether those cells are able to respond to cysLTs. No specific responses to LTE_4 or other leukotrienes were observed when intracellular signalling (calcium, cAMP) as well as cell activation (P-selectin expression and CCL5/RANTES release) was measured. In contrast, human platelets strongly responded to known $P2Y_{12}$ agonists and non-specific activators in those assays showing that cells were able to respond to appropriate stimulations implying that platelets are not a direct target for leukotrienes.

If platelets and $P2Y_{12}$ do not respond to LTE_4 as our data suggests, a question arises how observations from Paruchuri *et al.* on LTE_4 mediated pulmonary inflammation may be explained? Our hypothesis is that LTE_4 must activate specific receptors present on cells other than platelets, potentially structural cells such as endothelial cells, smooth muscle cells or tissue resident cells i.e. mast cells. Upon LTE_4 activation, such cells would produce (release) mediator(s) activating platelets or platelet-adherent leukocytes, facilitating cell adhesion to endothelium, cell activation and migration to tissue and as a result enhancing inflammatory responses. Platelet involvement in proinflammatory reactions, especially in pulmonary inflammation observed in asthma has been of increased interest recently. Clinical evidence has demonstrated increases in circulating platelets in atopic asthmatics, as well as increases in leukocyte-platelet aggregates after allergen challenge [24,25,26]. Recent advancements in the field have shown the direct importance of platelets in leukocyte recruitment and airway remodelling in allergic inflammation [14,27,28]. Therefore the reduction in LTE_4 mediated pulmonary inflammation seen in the study by Paruchuri *et al.* could be directly due to loss of $P2Y_{12}$ functionality rather than LTE_4 specific phenomenon [14]. However the studies of Paruchuri *et al.* and Maekawa *et al.* have elegantly highlighted that LTE_4 signalling can occur independently to the classical cysLT receptors, $CysLT_1$ and $CysLT_2$ [14,29] proving that LTE_4 preferentially signals via another as yet unidentified cysLT receptor.

In conclusion, our study strongly suggests that LTE_4 does not activate signalling either solely through $P2Y_{12}$ or through $P2Y_{12}$ being modulated by another receptor. The requirement to discover the true receptor for LTE_4 is still very apparent so that more effective anti-leukotriene therapies can be developed for the treatment of asthma.

Author Contributions

Conceived and designed the experiments: THL GW. Performed the experiments: HRF EF. Analyzed the data: HRF EF GW. Contributed reagents/materials/analysis tools: DJC. Wrote the paper: HRF THL DJC GW.

References

1. Peters-Golden M, Henderson WR (2007) Mechanisms of disease: Leukotrienes. N Engl J Med 357: 1841–1854.
2. Lynch K, O'Neill G, Liu G, Im D, Sawyer N, et al. (1999) Characterization of the human cysteinyl leukotriene $CysLT_1$ receptor. Nature 399: 789–793.
3. Heise CE, O'Dowd BF, Figueroa DJ, Sawyer N, Nguyen T, et al. (2000) Characterization of the human cysteinyl leukotriene 2 receptor. J Biol Chem 275: 30531–30536.
4. Woszczek G, Chen L-Y, Nagineni S, Alsaaty S, Harry A, et al. (2007) IFN-gamma induces cysteinyl leukotriene receptor 2 expression and enhances the responsiveness of human endothelial cells to cysteinyl leukotrienes. J Immunol 178: 5262–5270.
5. Woszczek G, Chen L-Y, Nagineni S, Kern S, Barb J, et al. (2008) Leukotriene D-4 induces gene expression in human monocytes through cysteinyl leukotriene type I receptor. J Allergy Clin Immunol 121: 215–221.
6. Lee TH, Woszczek G, Farooque SP (2009) Leukotriene E-4: Perspective on the forgotten mediator. J Allergy Clin Immunol 124: 417–421.
7. Diamant Z, Hiltermann JT, van Rensen EL, Callenbach PM, Veselic-Charvat M, et al. (1997) The effect of inhaled leukotriene D4 and methacholine on sputum cell differentials in asthma. Am J Respir Crit Care Med 155: 1247–1253.
8. Laitinen LA, Laitinen A, Haahtela T, Vilkka V, Spur BW, et al. (1993) Leukotriene E4 and granulocytic infiltration into asthmatic airways. Lancet 341: 989–990.
9. Gauvreau GM, Parameswaran KN, Watson RM, O'Byrne PM (2001) Inhaled leukotriene E(4), but not leukotriene D(4), increased airway inflammatory cells in subjects with atopic asthma. Am J Respir Crit Care Med 164: 1495–1500.
10. Soter NA, Lewis RA, Corey EJ, Austen KF (1983) Local effects of synthetic leukotrienes (LTC4, LTD4, LTE4, and LTB4) in human skin. J Invest Dermatol 80: 115–119.
11. Buckner CK, Fedyna JS, Robertson JL, Will JA, England DM, et al. (1990) An examination of the influence of the epithelium on contractile responses to peptidoleukotrienes and blockade by ICI 204,219 in isolated guinea pig trachea and human intralobar airways. J Pharmacol Exp Ther 252: 77–85.

12. Maekawa A, Kanaoka Y, Xing W, Austen KF (2008) Functional recognition of a distinct receptor preferential for leukotriene E4 in mice lacking the cysteinyl leukotriene 1 and 2 receptors. Proc Natl Acad Sci U S A 105: 16695–16700.
13. Nonaka Y, Hiramoto T, Fujita N (2005) Identification of endogenous surrogate ligands for human P2Y12 receptors by in silico and in vitro methods. Biochem Biophys Res Commun 337: 281–288.
14. Paruchuri S, Tashimo H, Feng CL, Maekawa A, Xing W, et al. (2009) Leukotriene E(4)-induced pulmonary inflammation is mediated by the P2Y(12) receptor. J Exp Med 206: 2543–2555.
15. Hollopeter G, Jantzen HM, Vincent D, Li G, England L, et al. (2001) Identification of the platelet ADP receptor targeted by antithrombotic drugs. Nature 409: 202–207.
16. Baurand A, Eckly A, Hechler B, Kauffenstein G, Galzi JL, et al. (2005) Differential regulation and relocalization of the platelet P2Y receptors after activation: a way to avoid loss of hemostatic properties? Mol Pharmacol 67: 721–733.
17. Chen LY, Eberlein M, Alsaaty S, Martinez-Anton A, Barb J, et al. (2011) Cooperative and redundant signaling of leukotriene B-4 and leukotriene D-4 in human monocytes. Allergy 66: 1304–1311.
18. Parmentier CN, Fuerst E, McDonald J, Bowen H, Lee TH, et al. (2012) Human T(H)2 cells respond to cysteinyl leukotrienes through selective expression of cysteinyl leukotriene receptor 1. J Allergy Clin Immunol 129: 1136–1142.
19. Brink C, Dahlen SE, Drazen J, Evans JF, Hay DW, et al. (2003) International Union of Pharmacology XXXVII. Nomenclature for leukotriene and lipoxin receptors. Pharmacol Rev 55: 195–227.
20. Offermanns S, Simon MI (1995) G-alpha(15) and G-alpha(16) couple a wide variety of receptors to phospholipase-C. J Biol Chem 270: 15175–15180.
21. Luttrell LM, Lefkowitz RJ (2002) The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. J Cell Sci 115: 455–465.
22. Altschul SF, Madden TL, Schaffer AA, Zhang JH, Zhang Z, et al. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389–3402.
23. Hardy AR, Jones ML, Mundell SJ, Poole AW (2004) Reciprocal cross-talk between P2Y(1) and P2Y(12) receptors at the level of calcium signaling in human platelets. Blood 104: 1745–1752.
24. Gresele P, Dottorini M, Selli ML, Iannacci L, Canino S, et al. (1993) Altered platelet- function associated with the bronchial hyperresponsiveness accompanying nocturnal asthma. J Allergy Clin Immunol 91: 894–902.
25. Kowal K, Pampuch A, Kowal-Bielecka O, DuBuske LM, Bodzenta-Lukaszyk A (2006) Platelet activation in allergic asthma patients during allergen challenge with Dermatophagoides pteronyssinus. Clin Exp Allergy 36: 426–432.
26. Yamamoto H, Nagata M, Tabe K, Kimura I, Kiuchi H, et al. (1993) The evidence of platelet activation in bronchial-asthma. J Allergy Clin Immunol 91: 79–87.
27. Pitchford SC, Yano H, Lever R, Riffo-Vasquez Y, Ciferri S, et al. (2003) Platelets are essential for leukocyte recruitment in allergic inflammation. J Allergy Clin Immunol 112: 109–118.
28. Pitchford SC, Riffo-Vasquez Y, Sousa A, Momi S, Gresele P, et al. (2004) Platelets are necessary for airway wall remodeling in a murine model of chronic allergic inflammation. Blood 103: 639–647.
29. Maekawa A, Kanaoka Y, Xing W, Austen K (2008) Functional recognition of a distinct receptor preferential for leukotriene E_4 in mice lacking the cysteinyl leukotriene 1 and 2 receptors. Proc Natl Acad Sci USA 105: 16695–16700.